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(54) Title: PROMOTERS FOR REGULATED GENE EXPRESSION

(57) Abstract: This invention provides nucleic acid sequences, vectors and host cells comprising regulatory regions associated with various promoters including a cyclin D1 promoter, a CD40L promoter, three HBV promoters (core, pre-S1 and HBV-X), a vancomycin-resistant enterococci (VRE) promoter, an androgen receptor promoter, a Her2 promoter, and a  $\beta$ -lactamase promoter. The invention further provides methods of regulating gene expression comprising the regulatory regions of such promoters.

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## PROMOTERS FOR REGULATED GENE EXPRESSION

Field of the Invention

The present invention relates to regulatory sequences within various promoters, and  
5 to heterologous nucleic acid constructs, vectors and transformation methods employing  
such sequences. The invention further relates to modified promoters and their use in  
regulated gene expression.

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Background of the Invention

- 10 Gene expression in prokaryotes and eukaryotes is a highly regulated process. Inappropriate expression (over-expression or under-expression) of "normal" or "healthy" genes is associated with many diseases and disease processes. Similarly, expression of mutated genes is also associated with many diseases. Controlling the expression of these genes is one of the ways through which diseases can be treated.
- 15 All genes contain transcriptional regulatory sequences upstream and downstream from the transcription start site. Transcription factors recognize and bind to transcriptional regulatory sequences and control the production of message transcribed from the gene. Transcriptional regulatory nucleic acid sequences involved in the regulation of gene expression include promoters, enhancers, and regulatory sequences to which
- 20 transcription factors or transcriptional regulatory proteins bind, which are required for initiation of transcription. Although transcriptional regulatory sequences are most frequently found just upstream of the transcription start site, they can also be found much further upstream, or on the 3' of the gene, or within the introns and exons that make up a gene.
- 25 A promoter is a region in a DNA sequence generally 1 to about 100 or 200 basepairs upstream of the transcription start site of a gene and typically contains or is adjacent to one or more transcription factor binding sites. An enhancer is a region in a DNA sequence that generally functions to increase transcription of a gene under its control. Enhancers are found upstream and/or downstream from the transcription start site. Enhancers can be located hundreds or even thousands of basepairs away from the transcription start site. Transcription factors bind to promoters and enhancers to regulate transcription.
- 30 35 The sequences of numerous transcriptional regulatory sequences are known in the art, some of which can be found in the "Eukaryotic Promoter Database" developed and maintained by members of the Bioinformatics Group of the ISREC (Swiss Institute

for Experimental Cancer Research), which is available on the Internet. However, absent a thorough analysis of the function of particular sequences found within a given promoter or enhancers, it is impossible to determine whether the particular sequences are important in regulating gene transcription. Once transcriptional regulatory 5 sequences have been identified, they may be utilized to regulate expression of the endogenous genes and may be incorporated into heterologous nucleic acid constructs for use in regulated expression of transgenes. Accordingly, it is of interest to identify and characterize the transcriptional regulatory regions of genes. Of particular interest are the regulatory regions of genes associated with various disease conditions, 10 examples of which are described below.

Mammalian cyclin D1 (CCND1, also named PRAD1 or BCL1) has applications to a number of cancers including but not limited to breast cancers, colon cancers and pancreatic cancers, and plays a critical role in regulating the G<sub>1</sub>/S checkpoint of the cell cycle of normal mature animal cells. (See Sherr, 1996)

15 CD40L ligand (CD40L) (also referred to as gp39, CD154, TRAP or T-BAM) plays a critical role in T cell dependent humoral immune responses by interacting with CD40, which provides a signal needed for T cell activation and recognition of antigen-MHC complexes by the T cell receptor.

20 Viral induced Hepatitis B (HBV) in humans is estimated to have infected 300 million people worldwide, with a small but significant number of infected individuals developing severe pathologic consequences, including chronic hepatic insufficiency, cirrhosis, and hepatocellular carcinoma. HBV-specific promoters involved in viral replication are therefore relevant to both therapy of HBV disease and regulated gene expression which is specific to liver cells.

25 The vancomycin resistance enzyme VanH has been associated with the recently observed increase in the incidence of infection and colonization with vancomycin-resistant enterococci (VRE). Therefore, regulated expression of VanH is relevant to treatment of VRE.

30 Prostate cancer is the most frequently diagnosed cancer in males in the United States. Current treatments for metastatic prostate cancer involve targeting the androgen receptor (AR) using surgical or chemical means. Regulated expression of the androgen receptor is relevant to treatment of prostate cancer.

Her2 (human epidermal growth factor receptor2; c-erbB2, neu) is a tyrosine kinase growth factor receptor which is overexpressed by breast cancer cells, ovarian

cancer cells and a variety of other cancer cells. Accordingly, regulated expression of Her-2 is relevant to modulating such overexpression.

The β-lactamase gene confers ampicillin resistance to *E. coli*. Accordingly, regulated expression of β-lactamase is relevant to modification of such antibiotic 5 resistance.

The present invention provides the sequences of the transcriptional regulatory regions of genes associated with various disease conditions together with a functional characterization of such sequences.

10 **Summary of the Invention**

The invention is directed to characterization of endogenous regulatory sites in the regulatory region of native gene promoters and their use in regulated gene expression.

In one aspect, the invention provides isolated nucleic acid sequences comprising the regulatory region of a cyclin D1 promoter, characterized by the ability to regulate 15 expression of a gene operably linked to a cyclin D1 promoter which includes the regulatory sequence. Exemplary sequences are presented as SEQ ID NO.:5, SEQ ID NO.:6 and SEQ ID NO.:8.

In another aspect, the invention provides isolated nucleic acid sequences comprising the regulatory region of a CD40L promoter, characterized by the ability to 20 regulate expression of a gene operably linked to a CD40L promoter which includes the regulatory sequence. Exemplary sequences are presented as SEQ ID NO.:12, SEQ ID NO.:13, SEQ ID NO.:14 and SEQ ID NO.:15.

In a further aspect, the invention provides isolated nucleic acid sequences comprising the regulatory region of an HBV promoter, characterized by the ability to 25 regulate expression of a gene operably linked to an HBV core, preS1 or X promoter which includes the regulatory sequence. Exemplary sequences are presented as SEQ ID NO.:20 and SEQ ID NO.:21 (core promoter); SEQ ID NO.:23 or SEQ ID NO.:24 (preS1 promoter); and SEQ ID NO.:26, SEQ ID NO.:27 and SEQ ID NO.:28 (HBV X promoter).

The invention also provides isolated nucleic acid sequences comprising the 30 regulatory region of a vancomycin-resistant enterococci (VRE) promoter, characterized by the ability to regulate expression of a gene operably linked to a VRE promoter which includes the regulatory sequence. Exemplary sequences are presented as SEQ ID NO.:32, SEQ ID NO.:33 and SEQ ID NO.:34.

The invention further provides isolated nucleic acid sequences comprising the 35 regulatory region of an androgen receptor (AR) promoter, characterized by the ability to

regulate expression of a gene operably linked to a AR promoter which includes the regulatory sequence. Exemplary sequences are presented as SEQ ID NO.:64, SEQ ID NO.:65 and SEQ ID NO.:66.

In another aspect, the invention provides isolated nucleic acid sequences  
5 comprising the regulatory region of a HER2 promoter, characterized by the ability to regulate expression of a gene operably linked to a HER2 promoter which includes the regulatory sequence. Exemplary sequences are presented as SEQ ID NO.:70, SEQ ID NO.:71 and SEQ ID NO.:72.

The invention further provides isolated nucleic acid sequences comprising the  
10 regulatory region of an androgen receptor beta lactamase (Bla) promoter, characterized by the ability to regulate expression of a gene operably linked to a Bla promoter which includes the regulatory sequence. Exemplary sequences are presented as SEQ ID NO.:77 or SEQ ID NO.:78.

In a related aspect the invention provides a vector comprising a promoter  
15 regulatory nucleic acid sequence for any one of: a cyclin D1 promoter, a CD40L promoter, three HBV promoters (core, pre-S1 and HBV-X), a vancomycin-resistant enterococci (VRE) promoter, an androgen receptor promoter, a Her2 promoter, and a β-lactamase promoter, as described above.

The vector may be an expression vector which includes the promoter regulatory  
20 sequence operably linked to a promoter and control sequences recognized by a host cell transformed with the vector; and a transgene encoding a gene product, e.g., a reporter gene.

A host cell comprising such a vector, e.g., a prokaryotic cell, a eukaryotic cell, or a  
25 mammalian cell is also provided by the invention. A host cell transformed with such a vector may be used in a method for regulating expression of a transgene and detecting the expression thereof, e.g., by exposing the cell to a cellular factor or a DNA binding compound which interacts with the promoter regulatory sequence.

#### Brief Description of the Figures

30 Figure 1A presents the sequence of the HBV core promoter.

Figure 1B presents the sequence of the HBV pre-S1 promoter region with the sequences of various DNA response elements (HNF1, HNF3, Sp1 and TBP) indicated as underlined with sequence locations indicated in the figure.

35 Figure 2 depicts the results of a hybridization stabilization assay (HSA) with various HBV preS1 promoter constructs indicating the binding preference of a test

compound, the netropsin dimer, 21x, for the HNF3-wt, TBP-wt, TBP-mut, HNF-1-wt, HNF1-m and HNF1-21x sequences, indicated in the figure.

Figure 3 presents the sequence of the HBV X promoter region with the sequences of various DNA response elements (NF1, 2c, EF-C, NF-1 and X-PBP) indicated as underlined in the figure.

5 Figure 4 presents the sequence of the wild type cyclin D1 promoter from -1745 to +155, which corresponds to nucleotides 316 to 2161 of GenBank Accession No. L09054.

Figures 5A to C present the sequence of the full-length human CD40L sequence numbered from nucleotide 1 to 2395, wherein nucleotides 10 to 1919 correspond to the 10 human CD40L promoter sequence identified as -1860 to +49.

Figure 6 presents the sequence of the wild type vanH promoter.

Figure 7 presents the sequences of vanH promoter mutants M2-M21, wherein each group of 10 nucleotides in the original vanH promoter sequence shown in the figure was replaced with the mutant sequence, e.g., in M2 the CCCGGGGGGC sequence was 15 inserted in place of the wild type TAATTTTTTA sequence.

Figures 8A to C present the sequence of the wild type androgen receptor promoter from -6000 to +1100.

Figure 9 presents the sequence of the wild type Her2 promoter.

20 **Detailed Description of the Invention**

**I. Definitions**

As used herein, the term "polynucleotide" refers to a polymeric molecule having a backbone that supports bases capable of hydrogen bonding to typical polynucleotides, where the polymer backbone presents the bases in a manner to permit such hydrogen bonding in a sequence specific fashion between the polymeric molecule and a typical polynucleotide (e.g., single-stranded DNA). Such bases are typically adenosine, guanosine, cytosine, thymidine, uracil and inosine. Polymeric molecules include double and single stranded ribonucleic acids (RNA) and deoxyribonucleic acids (DNA), and may include polymers having backbone modifications such methylphosphonate linkages.

25 30 As used herein, a nucleic acid may be double stranded, single stranded, or contain portions of both double stranded or single stranded sequence. The depiction of a single strand also defines the sequence of the other strand and thus also includes the complement of the sequence.

As used herein, the term "recombinant nucleic acid" refers to a nucleic acid, originally formed *in vitro*, in general, by the manipulation of the nucleic acid in a form not normally found in nature.

A "heterologous nucleic acid construct" has a sequence portion that is not native to the cell in which it is expressed. Heterologous, with respect to a control sequence/coding sequence combination refers to a control sequence (*i.e.*, promoter or enhancer) and a coding sequence or gene combination, that is not found together in nature, in other words, the promoter does not regulate the expression of the same gene in the heterologous nucleic acid construct and in nature. Generally, heterologous nucleic acid sequences are not endogenous to the cell or part of the genome in which they are present and have been added to the cell, by transfection, microinjection, electroporation, or the like. Such a heterologous nucleic acid construct may also be referred to herein as an "expression cassette".

As used herein, the term "sequence identity" means nucleic acid or amino acid sequence identity between two or more sequences, when aligned using a sequence alignment program. Sequence searches are preferably carried out using the BLASTN program when evaluating the % identity of a given nucleic acid sequence relative to nucleic acid sequences in the GenBank DNA Sequences and other public databases. The BLASTX program is preferred for searching nucleic acid sequences which have been translated in all reading frames against amino acid sequences in the GenBank Protein Sequences and other public databases. Both BLASTN and BLASTX are run using default parameters with an open gap penalty of 11.0, and an extended gap penalty of 1.0, and utilize the BLOSUM-62 matrix. [See, Altschul *et al.*, 1997.]

The term "% homology" is used interchangeably herein with the term "% identity" and refers to the level of identity between two sequences, *i.e.* 70% homology means the same thing as 70% sequence identity as determined by a defined algorithm, and accordingly a homologue of a given sequence has at least about 70%, preferably about 80%, more preferably about 85%, even more preferably about 90% sequence identity over a length of the given sequence.

A preferred alignment of selected sequences in order to determine "% identity" between two or more sequences, is performed using the CLUSTAL-W program in MacVector version 6.5, operated with default parameters, including an open gap penalty of 10.0, an extended gap penalty of 0.1, and a BLOSUM 30 similarity matrix.

A nucleic acid sequence is considered to be "selectively hybridizable" to a reference nucleic acid sequence if the two sequences specifically hybridize to one another

under moderate to high stringency hybridization and wash conditions. Exemplary conditions include hybridization conducted as described in the Bio-Rad Labs ZetaProbe manual (Bio-Rad Labs, Hercules, CA). For example, hybridization is conducted in 1mM EDTA, 0.25 M Na<sub>2</sub>HPO<sub>4</sub> and 7% SDS at 60° C, followed by washing in 1mM EDTA,

- 5 40mM NaPO<sub>4</sub>, 5% SDS, and 1mM EDTA, 40 mM NaPO<sub>4</sub>, 1% SDS. Hybridization conditions are further recited in Ausubel FM et al., 1993.

As used herein, the term "vector" refers to a nucleic acid construct designed for transfer between different host cells. An "expression vector" refers to a vector that has the ability to incorporate and express heterologous DNA fragments in a foreign cell. Many 10 prokaryotic and eukaryotic expression vectors are commercially available. Selection of appropriate expression vectors is within the knowledge of those having skill in the art.

As used herein, the term "plasmid" refers to a circular double-stranded (ds) DNA construct used as a vector, which forms an extrachromosomal self-replicating genetic element in many bacteria and some eukaryotes.

- 15 As used herein, the term "gene" means the segment of DNA involved in producing a polypeptide, which may or may not include regions preceding and following the coding region. For example, 5' untranslated (5' UTR) or "leader" sequences and 3' UTR or "trailer" sequences, as well as intervening sequences (introns) between individual coding segments (exons), may or may not be included in the DNA segment designated as the 20 gene.

As used herein the term "transgene" refers to the portion of a heterologous nucleic acid construct, expression cassette or vector which comprises the coding sequence for a polypeptide, wherein the gene is associated with other components, i.e., a promoter with which it is not normally associated in nature.

- 25 As used herein, the term "DNA response element" may be used interchangeably with the term "regulatory promoter sequence" and refers to the DNA binding site or sequence for a transcriptional regulatory protein, which may be the same as, overlapping, or adjacent to, a compound-binding sequence.

- 30 As used herein, the terms "compound binding sequence", "compound binding site", "ligand binding sequence", and "ligand binding site" are used interchangeably and refer to the portion of a DNA sequence with which a compound, ligand, or molecule interacts resulting in the modified binding of a transcriptional regulatory protein to its DNA binding site (or DNA response element). In some cases, the compound, ligand, or molecule may also be designated a compound or inducer. The "compound-binding 35 sequence" or equivalent is in the vicinity of the DNA response element for transcriptional

regulatory protein and may be adjacent (*i.e.*, flanking), overlapping, or the same as the DNA binding site for a transcriptional regulatory protein.

- As used herein, the term "promoter" refers to a sequence of DNA that functions to direct transcription of a gene that is operably linked thereto. The promoter may or may not include control sequences (also termed "transcriptional and translational regulatory sequences"), involved in expression of a given gene product. In general, transcriptional and translational regulatory sequences include, but are not limited to, the promoter sequence, include the DNA response element for a transcriptional regulatory protein, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. The promoter may be native or non-native to the cell in which it is found.

As used herein, the terms "regulatable promoter", "inducible promoter" and "switchable promoter", are used interchangeably and refer to any promoter the activity of which is affected by a *cis* or *trans* acting factor.

- A eukaryotic gene control region consists of a promoter plus regulatory DNA sequences (to which transcriptional regulatory proteins bind). As used herein, the term "regulatory promoter sequence" generally refers to a sequence within the control region of a gene and to which transcriptional regulatory proteins bind, resulting in transcriptional activation or repression. Native forms of such regulatory promoter sequences are generally located 5' to the promoter elements of the gene control region.

- As used herein, the terms "transcriptional regulatory protein", "transcriptional regulatory factor" and "transcription factor" may be used interchangeably with the term "DNA-binding protein" and refer to a cytoplasmic or nuclear protein that binds a DNA response element and thereby transcriptionally regulates the expression of an associated gene or genes. Transcriptional regulatory proteins generally bind directly to a DNA response element, however in some cases binding to DNA may be indirect by way of binding to another protein which in turn binds to, or is bound to the DNA response element.

- As used herein, the term "operably linked" relative to a recombinant DNA construct or vector means a nucleotide component of the recombinant DNA construct or vector is in a functional relationship with another nucleotide component of the recombinant DNA construct or vector. For example, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation.
- Generally, "operably linked" means that the DNA sequences being linked are contiguous,

and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous.

As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the information contained in a given DNA sequence. The process 5 includes both transcription and translation.

As used herein, the term "modulated expression" refers to a change in transcription and translation, which may represent an increase or a decrease in the amount of a given gene product.

A host cell has been "transformed" by exogenous or heterologous DNA when the 10 DNA has been introduced into the cell. Transformation may or may not result in integration (covalent incorporation) into the chromosomal DNA of the cell. For example, in eukaryotic cells such as yeast and mammalian cells, the transfected DNA may be maintained on an episomal element such as a plasmid.

As used herein, the terms "stably transformed", "stably transfected" and 15 "transgenic" refer to cells that have a non-native (heterologous) nucleic acid sequence integrated into the genome. Stable transformation is demonstrated by the establishment of cell lines or clones comprised of a population of daughter cells containing the transfecting DNA.

In some cases, "transformation" is not stable, *i.e.*, it is transient. In the case of 20 transient transformation, the exogenous or heterologous DNA is expressed, however, the introduced sequence is not integrated into the genome.

As used herein, the term "co-transformed" refers to a process by which two or more recombinant DNA constructs or vectors are introduced into the same cell. "Co-transformed" may also refer to a cell into which two or more recombinant DNA constructs 25 or vectors have been introduced.

As used herein, the term "sequence preferential binding" refers to the binding of a molecule to DNA in a manner that indicates a preference for binding to a certain DNA sequence relative to others.

As used herein, the term "sequence specific binding" refers to the binding of a 30 molecule to DNA in a manner that indicates a strong binding preference for a particular DNA sequence.

As used herein, the term "sequence-dependent binding" refers to the binding of molecules to DNA in a manner that is dependent upon the target nucleotide sequence. Such binding may be "sequence-preferential" or "sequence-specific".

As used herein, the term "inhibit binding" relative to the effect of a given concentration of a particular compound on the binding of a transcriptional regulatory protein to its DNA response element refers to a decrease in the amount of binding of the transcriptional regulatory protein to its DNA response element relative to the amount of binding in the absence of the same concentration of the particular compound, and includes both a decrease in binding as well as a complete inhibition of binding.

5 As used herein, the terms "compound", "molecule", "ligand" and "inducer" are used interchangeably and refer to molecules or ligands characterized by sequence-preferential or sequence-specific binding to DNA at a sequence which is adjacent (i.e., flanking), overlapping, or the same as, the DNA binding site for a transcriptional regulatory protein.

10 As used herein, the terms "modulate" and "modify" are used interchangeably and refer to a change in biological activity. Modulation may relate to an increase or a decrease in biological activity, binding characteristics, or any other biological, functional, or immunological property of the molecule.

15 As used herein, the term "regulate gene expression" relative to a promoter of the invention means the promoter has the ability to increase or decrease the expression of, and may be used to modulate the level of expression of a gene operably linked thereto.

20 As used herein, the terms "native", "natural" and "wild-type" relative to a particular nucleic acid sequence, trait or phenotype refers to the form in which that nucleic acid sequence, trait or phenotype is found in nature.

25 As used herein, the term "exposure of said cell" relative to a cellular factor or compound which may interact with a cell that comprises a regulatory promoter sequence of the invention refers to both external and internal exposure. In the case of exposure to a cellular factor, the factor may be native (endogenous) or exogenously provided.

## II. Regulated Gene Expression using Promoters of the Invention

The promoter elements of the present invention find utility in the regulated expression of genes, both native and heterologous.

30 In order to accomplish such regulated gene expression the regulatory components of a promoter of interest must be identified and characterized.

35 This is accomplished by the combination of identifying and characterizing the sequence of promoter components involved in the control of gene transcription and correlating such structural (sequence) components with a functional analysis of gene expression using the promoter.

In general, to determine if a particular DNA sequence is involved in the regulation of gene expression, a putative regulatory sequence is selected and operably linked to a reporter sequence in a heterologous nucleic acid construct which is then introduced into a cell, then the reporter activity is determined. For example, the expression of luciferase, a  
5 gene originally isolated from the firefly that emits a photon in the presence of the substrate luciferin and ATP is easily monitored using a luminometer.

In one application of such regulated gene expression, compound binding sequences, located in the vicinity of the DNA response element for a transcriptional regulatory protein are incorporated into promoter constructs and used to regulate  
10 expression of a gene under the control of a given promoter. The compound binding sequences may be native or introduced.

In another exemplary embodiment, the binding of a compound in the vicinity of (i.e., directly, adjacent to, or overlapping) the DNA response element for a transcriptional regulatory protein provides a means to modulate transcription of a native gene operably  
15 linked to the DNA response element.

The identification and characterization of the regulatory regions of a promoter and using that information to design constructs which have one or more compound binding sequences in the vicinity of the DNA response element for a given transcriptional regulatory protein provides a means to regulate expression of native genes *in vivo* in a  
20 cell. In such cases, providing the compound to a cell and the binding of the compound to a compound binding sequence within the regulatory region of a promoter results in regulated expression of a native gene under the control of that promoter.

In another exemplary embodiment, the binding of a compound in the vicinity of (i.e., directly to, adjacent, or overlapping) the DNA response element for a transcriptional  
25 regulatory protein provides a means to modulate transcription of a transgene operably linked thereto. Any DNA binding compound that modulates the binding of a transcriptional regulatory protein to its DNA response element can be utilized to modulate expression of a transgene under the control of a promoter based on the present invention. The presence of a native or introduced compound-binding sequence in the vicinity of the DNA  
30 response element for a transcriptional regulatory protein permits a wide selection of compounds effective to regulate the expression of genes operably linked to a promoter wherein the promoter includes the DNA response element.

It will be understood that a promoter of the invention may include a minimal promoter element and an introduced DNA response element, or the promoter itself may  
35 contain a DNA response element. In general, the DNA response element or regulatory

promoter sequence refers to the sequence to which transcriptional regulatory proteins bind and may or may not be considered part of the promoter.

In some cases, the nucleic acid sequence in the vicinity of the DNA response element will include a sequence that is the preferred or specific binding site for a DNA binding compound.

In other cases, the promoter sequence in the vicinity of the DNA response element will be modified to include one or more preferred binding sequences for a DNA-binding compound resulting in a regulatable promoter construct.

For example, the promoter may include one or more compound binding sequences in the vicinity of the DNA response element, as exemplified by an 8 to 20 or more bp "AT-rich" sequence which is a preferred binding preferred binding sequence for the netropsin dimer, "21x".

A transcriptional regulatory protein/DNA response element/compound binding sequence combination together with a compound which preferentially or specifically binds to that compound binding sequence may be useful for regulated expression of a transgene under the control of any of the promoters described herein. However, in some cases, the transcriptional regulatory protein/DNA response element/compound binding sequence combination and the compound which preferentially or specifically binds to that compound binding sequence is specific to a given promoter.

Compounds for use in regulating expression of a transgene under the control of a particular promoter are generally pre-selected based on the ability to regulate the expression of a transgene under the control of a given promoter.

Exemplary pre-screening assays include, but are not limited to, DNA binding assays; protein displacement assays; DNA footprinting, etc. As set forth herein, such assays may be carried out using various techniques known in the art.

In one embodiment, compounds for use in regulating gene expression are pre-selected for DNA-binding and transcriptional regulatory protein displacement. Exemplary pre-screening assays include various forms of the Merlin™ assay, e.g., co-owned U.S. Pat. Nos. 5,306,619, 5,693,463, 5,716,780, 5,726,014, 5,744,131, 5,738,990, 5,578,444, 5,869,241, expressly incorporated reference herein.

In another embodiment, compounds are pre-selected in a nucleic acid ligand interaction assay, such as that described in PCT Publication No. WO 00/15848, or another nucleic acid binding assay known to those of skill in the art.

### III. Promoter Isolation and Characterization

The promoters described herein were isolated and characterized employing methods generally known in the art, including, but not limited to, walking upstream from the coding sequence of a known gene to identify regulatory sequences, analysis and 5 characterization of previously identified promoter sequences by linker scanner mutation and site directed mutagenesis.

In some cases, promoter sequences are obtained by walking upstream in a PCR-accessible genomic library (e.g., using GenomeWalker, Clontech) using primers designed based on a known coding or other sequence. Sequential upstream walks are used to 10 generate longer DNA sequences, extended at the 5' end in order to identify regulatory sequences. The sequence obtained from a first walk is used to design primers for a second upstream walk, etc.

In other cases, the full sequence of a particular promoter for which the regulatory 15 sequences are described herein, was known in the art. However, in such cases the characterization of the promoter was not known prior to the present invention. In other words, the present invention represents identification and characterization of sequences critical to promoter activity.

In some cases, a series of promoters were constructed by introducing mutations in one or more regions of the promoter sequence followed by evaluation of the activity profile 20 of the mutated promoters.

### IV. Promoter Activity Screening

Exemplary assays for evaluation of promoter activity include, but are not limited to, DNA binding assays useful for detection of the binding of a transcriptional regulatory 25 protein to the DNA response element of a promoter; protein displacement assays, such as gel mobility shift assays, competitive binding assays and DNA footprinting, etc. Such assays may be carried out using various techniques known in the art.

Gel mobility shift assays may be used to determine the effect of a compound on the binding of a transcriptional regulatory protein to the DNA response element within a 30 given promoter, based on the change in size (and corresponding mobility on a gel) of the DNA/protein complex relative to the DNA alone.

DNA footprinting may be used to characterize the DNA response element of a given promoter for a transcriptional regulatory protein based on the stability of a promoter/transcriptional regulatory protein complex to nuclease degradation. The main application 35 of this approach has been for DNA footprinting (a method used to identify the DNA

sequence to which particular transcriptional regulatory proteins bind). Various techniques for DNA footprinting are known in the art.

Competitive hybridization-stabilization binding assay (HSA)

The binding preference of compounds to critical sequences in the promoters of the invention has been examined using a competitive hybridization-stabilization binding assay (HSA). In the HSA, a nucleotide sequence of interest is represented in an oligonucleotide duplex, and the duplex is tested for its ability to compete with an indicator oligonucleotide duplex which is known to bind the test molecule with a certain degree of affinity. The indicators may be rich in AT bases and labeled with either a fluorescent probe or a quencher moiety on each of the two strands. The binding of the compound to the indicator stabilizes the duplex formation allowing the fluorescence to be quenched. If the compound prefers the test sequence (competitor) more than the indicator, it is less available to stabilize the indicator duplex and thus quenching is reduced. Therefore, a higher fluorescence signal implies a higher degree of binding preference to the test sequence relative to the indicator.

In one example involving the cyclin D1 promoter, the hybridization stabilization assay employs a 12bp DNA duplex as an indicator for binding, wherein one strand of the duplex (CTTTATTATTTT) is 5' labeled with fluorescein, and the complementary strand is 5' labeled with a dabsyl quenching molecule (AAAATAATAAG-3'). When the two strands are mixed together with a DNA-binding molecule, which can stabilize the duplex form, the signal from the fluorescein is quenched by the dabsyl on the complementary strand. Various cold competitor duplexes can then be added to see whether they provide preferred binding sites for the DNA-binding compound. If the competitor DNA binds the DNA-binding molecule, the DNA-binding molecule is titrated away from the indicator duplex resulting in destabilization of the indicator duplex and as the strands separate, quenching is diminished and fluorescence increases.

Promoter-walk analysis Typically, a full promoter sequence is presented in blocks of 15 nucleotides as the competitor in a HSA. To cover the entire promoter, stretches of 15-mers are blocked in an overlapping manner so that neighboring blocks differ by two nucleotides. An increase in fluorescence in the HSA implies a preference in binding.

RNase protection The effect of a modified DNA sequence on RNA transcription may be measured directly using an assay that includes either RNase protection or Northern analysis to monitor mRNA levels. RNase protection is a method of quantitating RNA based on its ability to form a nuclease resistant hybrid with a labeled probe. With more RNA, more probe can be protected. If only part of the probe hybridizes to the RNA

of interest (*i.e.*, the probe has 5' or 3' regions that are not homologous to the RNA of interest), then only part of the probe is protected. The protected probe and the intact probe will migrate at different rates when subjected to gel electrophoresis. Protection of a fragment of a unique and predictable size indicates specificity. The probe can be either  
5 an RNA or a DNA probe.

Linker Scanning Mutagenesis is a procedure in which short sequences of a DNA (*i.e.* sequences 5' to a known promoter) are substituted with DNA containing one or more restriction sites, usually using a PCR based mutagenesis approach.

Reporter Constructs

10 A reporter construct is generally used in a cell-based *in vitro* assay to confirm promoter activity and the regulated expression of a transgene by a promoter.

In one embodiment, the luciferase reporter gene is used to evaluate regulatable gene expression *in vitro* in cell culture. However, any reporter gene known to those of skill in the art may also be used. It is preferable that expression of the selected reporter  
15 gene be readily detected and quantitated in order to quickly evaluate numerous modified regulatory sequences. Such reporter constructs provide a means to evaluate the ability to regulate gene expression by a given promoter, *e.g.*, by targeting with a DNA-binding compound. Once the ability of a given promoter to regulate gene expression has been demonstrated in a cell-based assay using a reporter construct, the genetic construct may  
20 be readily modified to include a transgene of interest, such as a therapeutic gene, recombinant protein-encoding gene or drug resistance gene, in place of the reporter gene. Such modifications may be made using techniques routinely employed by those of skill in the art.

25 V. Cyclin D1 Promoter

Cyclin D1 (CCND1) is a regulatory protein overexpressed in many carcinomas. Cyclin D1 acts by binding to and regulating the cyclin dependent kinases CDK4 and CDK6. CCND1 gene expression is low in quiescent cells (in G<sub>0</sub>) but is induced as cells respond to growth factors and enter the cell cycle leading to an increase in active cyclin  
30 D1-CDK4/CDK6 complexes.

Rapid cell cycling irrespective of appropriate growth signals and failure to respond to growth inhibition signals such as contact inhibition are characteristics of cancer cells. Inappropriate expression of cyclin D1 during chromosomal inversion, translocation or amplification has been characterized in a variety of tumor cells (Hall *et al.*, 1996; Sherr,  
35 1996). Cyclin D1 gene overexpression is also seen in many tumors without gross

chromosomal rearrangements or amplification of the cyclin D1 gene. In fact, overexpression of cyclin D1 is seen in 50% of primary breast carcinomas, in 30% of adenocarcinomas of the colon cells (Hall *et al.*, 1996), in familial adenomatous polyposis (Zhang *et al.*, 1997) as well as in many cases of pancreatic cancer (Gansauge *et al.*, 1997).

In addition, transgenic mice that overexpress the cyclin D1 gene in mammary epithelium show mammary hyperplasia and develop mammary adenocarcinomas (Wang *et al.*, 1994). Overexpression of cyclin D1 in cultured cells results in early phosphorylation of pRB retinoblastoma protein (Sherr, 1993), shortening of the G1 phase and makes these cells growth factor independent (Jiang *et al.*, 1993; Quelle *et al.*, 1993; Resnitzky *et al.*, 1994). When injected into nude mice these cells produce tumors (Jiang *et al.*, 1993).

The link between inappropriate expression of cyclin D1 and tumorigenesis indicates that cyclin D1 is a good target for therapeutic intervention. Cyclin D1 antisense molecules have been shown to reduce the neoplastic phenotype of human esophageal, colon and pancreatic cancer cells overexpressing cyclin D1 in culture as well as the ability of these cells to produce tumors in mice (Zhou *et al.*, 1995; Arber *et al.*, 1997; Kornmann *et al.*, 1998). In these studies antisense technology was used to specifically inhibit cyclin D1 mRNAs.

Accordingly, regulated expression of cyclin D1 finds utility in cancer and other therapies. The present invention is based on CCND1 promoter analysis and identification of DNA response elements within the cyclin D1 promoter that are involved in regulation of gene expression, when under the control of the cyclin D1 promoter.

The human CCND1 gene has been previously cloned and sequenced (Motokura *et al.*, 1991; Withers *et al.*, 1991; Xiong *et al.*, 1991). An upstream promoter sequence of the CCND1 gene has also been cloned and sequenced (Herber *et al.*, 1994a, 1994b; Philipp *et al.*, 1994). The CCND1 promoter sequence may be found in GenBank at Locus HUMPRDA1A (Motokura *et al.*, 1993).

Potential Sp1, E2F, CRE, Oct1, Myc/Max, AP-1, Egr, NF $\kappa$ B, STAT5, Ets, PRAD and TCF/LEF sites have been previously identified in the cyclin D1 promoter (Motokura *et al.*, 1993; Herber *et al.*, 1994; Philipp *et al.*, 1994; Hinz *et al.*, 1999; Matsumura *et al.*, 1999; Shtutman *et al.*, 1999; and Tetsu *et al.*, 1999). Several of these sites have been demonstrated to play a role in cyclin D1 regulation in various cell lines (Philipp *et al.*, 1994; Albanese *et al.*, 1995; Watanabe *et al.*, 1996; Yan, *et al.*, 1997; Watanabe *et al.*, 1998; Beier *et al.*, 1999; Hinz *et al.*, 1999; Matsumura *et al.*, 1999; Shtutman *et al.*, 1999; and Tetsu *et al.*, 1999).

The CRE region of the CCND1 promoter (nucleotides -52 to -45) has previously been identified as important for cyclin D1 expression in various cell types (Beier *et al.*, 1999; Tetsu *et al.*, 1999; Phillip *et al.*, 1994; Lee *et al.*, 1999). In particular, the CRE promoter element has been demonstrated to be required for basal expression of the cyclin  
5 D1 gene in MCF7 cells.

Although the prior art includes some analysis of the cyclin D1 promoter, the prior art does not indicate appropriate targets for regulated gene expression using the cyclin D1 promoter. One aspect of the present invention is directed to modulating cyclin D1 expression in cancer cells that overexpress the gene, based on particular sequences  
10 identified as targets for regulation.

Analysis of transcription factor binding sites in the cyclin D1 promoter was carried out to identify portions of the cyclin D1 promoter that can be used to regulate the expression of a gene operably linked to the cyclin D1 promoter. An extensive promoter analysis was performed in a variety of different cancer cell lines that overexpress cyclin  
15 D1 and important transcription factor binding sites were identified, as detailed in Example 1.

A 1900-bp fragment of the human cyclin D1 promoter was PCR amplified from genomic DNA and subcloned into the vector pGL3-basic (Promega) to form a reporter construct. A series of modified promoters were made and promoter activities compared to that of the full-length (-1745) cyclin D1 promoter (Fig. 4) following transfection into asynchronous MCF7 human breast carcinoma cells, which overexpress cyclin D1, in order to identify important regulatory regions of the promoter. Some constructs were further evaluated in another cyclin D1 overexpressing breast carcinoma cell line (ZR75); in a breast cell line (HMEC) that expresses cyclin D1 normally; in a cyclin D1  
25 overexpressing colon cancer cell line (HCT116); and an overexpressing pancreatic cancer cell line (PANC-1).

The various modified promoter constructs include 5' deletions, site-directed mutagenesis of the AP1, CRE, E2F, SP1 and Oct1 sites, and mutants prepared using linker-scanning mutagenesis of the proximal promoter generated using the QuickChange  
30 mutagenesis system.

The results provided herein indicate that the regulatory sequences presented as SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:9 find utility in regulating the expression of autologous or heterologous genes operably linked to a cyclin D1 promoter comprising one or more of the regulatory sequences.

## VI. CD40 ligand (CD40L)

CD40 ligand or CD40L (also referred to as gp39, CD154, TRAP or T-BAM) plays a critical role in T cell dependent humoral immune responses. CD40L interacts with CD40, which is expressed on the surface of antigen presenting cells (APCs; Ochs *et al.*, 1994; Foy *et al.*, 1996; Grewal *et al.*, 1996). Antigen presenting cells process antigens and present them on their surface in combination with major histocompatibility complex (MHC) molecules. This provides one signal necessary for T cell activation and recognition of an antigen-MHC complex by the T cell receptor which triggers the transient expression of the membrane bound cytokine CD40L on activated CD4+ helper T cells. Interaction between CD40 and CD40L is necessary for B cell activation and isotype switching. The binding of CD40L to CD40 induces the expression of the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) on APCs which in turn, bind to CD28 on T cells providing the second costimulatory signal necessary for T cell activation. Engagement of the T cell receptor by antigen-MHC in the absence of the second signal produces T cell anergy. A human genetic defect in the CD40L gene causes the X-linked immunodeficiency disorder called hyper-IgM syndrome (Allen *et al.*, 1993; Aruffo *et al.*, 1993; DiSanto *et al.*, 1993; Korthauer *et al.*, 1993). Affected individuals either fail to express CD40L or express CD40L incapable of binding to CD40 resulting in significantly reduced T cell-dependent humoral immune responses and an absence of isotype class switching.

Targeting the CD40L promoter therefore has implications to a number of autoimmune disorders, including but not limited to, multiple sclerosis (MS), systemic lupus erythematosus (SLE), graft-vs-host disease (GVHD) and rheumatoid arthritis. (See, e.g., Buhlmann *et al.*, 1996; Biancone *et al.*, 1999). In addition, there is evidence that inhibiting CD40L expression can contribute to long term transplantation tolerance (Larsen *et al.*, 1996; Kirk *et al.*, 1997; Hancock *et al.*, 1998; Niimi *et al.*, 1998). Further, targeting CD40L with specific monoclonal antibodies has been shown to increase the effectiveness of adenovirus vector based gene therapy (Yang *et al.*, 1996; Kay *et al.*, 1997).

The human CD40L gene has been cloned (Graf *et al.*, 1992; Hollenbaugh *et al.*, 1992; Spriggs *et al.*, 1992; Gauchat *et al.*, 1993; Shimadzu *et al.*, 1995). The CD40L promoter sequence contains several potential transcription factor binding sites: AP-1 (1570 to 1577; 1867 to 1938), GMCSF (1040 to 0145; 1343 to 1350; 1689 to 1696; 1840 to 1862),  $\alpha$  IRE (1291 to 1295; 1359 to 1366; 1397 to 1404; 1589 to 1593; 1701 to 1705; and 1803 to 1807), TCF1 (1603 to 1606; 1731 to 136), GATA-1 (1643 to 1647),

CRE 2(1209 to 1216), γ INF2 (1188 to 1195), NF-IL6 (815 to 819) and NFκB (737-743) as identified by sequence analysis (GenBank Accession No. D31793).

- In order to characterize the CD40L promoter, the full-length human CD40L promoter from -1860 to +49 (SEQ ID NO:1) was PCR amplified and cloned into the
- 5 firefly luciferase reporter plasmid pGL3-basic, as detailed in Example 2. A series of 5' CD40L promoter deletions and specific mutations were prepared, PCR amplified and cloned into the firefly luciferase reporter plasmid pGL3-basic, the authenticity of all clones verified by DNA sequencing and promoter activity of the 5' deletion constructs compared to that of the full-length (-1860) CD40L promoter following transfection into
- 10 normal expanded T cells and activation with PMA and ionomycin (Example 2).

The results indicate that at least four regions of the CD40L promoter are critical to expression in activated T cells, including a site near nucleotide position -306, the specific mutation of which resulted in a 4-fold down regulation of CD40L promoter activity factor binding at the site. (See Example 2)

- 15 A second promoter region that plays a role in controlling CD40L expression is the sequence between nucleotides -230 and -211 (SEQ ID NO:13), based on deletion of the region which resulted in a 6.7-fold reduction in promoter activity.

- 20 A third region important to CD40L promoter expression in activated normal human T cells is found between -230 and -196 (SEQ ID NO:14), based on deletion of the -230 to -211 region, which resulted in an 6.7-fold downregulation of CD40L promoter activity, and site specific mutations of -220 to -215, -214 to -209, -208 to -203 or -202 to -197, which resulted in a 2.5 to 4-fold down regulation of promoter activity. A T cell-specific, sequence-specific factor was demonstrated to bind in the -206 to -201 region based on the results of *in vivo* footprinting analysis.

- 25 A fourth region identified in the CD40L promoter as important for expression in activated normal human T cells is found between -77 and -40 (SEQ ID NO:15) based on the expression level of deletion mutants, wherein an internal deletion of -72 to -49 or -61 to -40 resulted in a 25-fold or 40-fold downregulation respectively. In addition, specific mutations in the composite AP-1/-66 NF-AT site together with a previously unidentified
- 30 site located between -48 and -54 indicates a contribution to transcriptional activation through the -48 to -54 site.

It will be appreciated that some CD40L promoter regions may bind more than one transcription factor, as further discussed in Example 2. It will further be appreciated that targeting a DNA-binding compound to a regulatory region of the CD40L promoter

described herein, provides a means to inhibit CD40L promoter-mediated transcription through modulation of transcription factor-DNA interactions.

The results provided herein indicate that the regulatory sequences presented as SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14 and SEQ ID NO:15 find utility in regulating 5 the expression of autologous or heterologous genes operably linked to a CD40L promoter comprising one or more of the regulatory sequences.

## VII. Hepatitis B (HBV)

10 Viral induced Hepatitis B in humans is caused by infection with HBV, which is estimated to have infected 300 million people worldwide. A small but significant portion of the infected individuals develop severe pathologic consequences, including chronic hepatic insufficiency, cirrhosis, and hepatocellular carcinoma, with one million deaths per year caused by HBV infection worldwide.

15 Vaccination is an effective preventive measure, however, there is no cure for the disease, and at present there is no effective treatment specific to acute hepatitis B. Currently, chronic hepatitis B is treated with interferons (*i.e.*, interferon-alpha) and nucleoside analogs (*i.e.*, lamivudine "3TC").

20 HBV was initially cloned in the 1970s (Robinson *et al.*, 1974; Sattler *et al.*, 1979; Summers *et al.*, 1975). Human hepatoma cell lines (HepG2 and HuH6) have HBV stably integrated within the cellular genome. These cells can support HBV replication and release virus-like particles into the tissue culture media. See, e.g., See MA *et al.*, 1987; Lander *et al.*, 1997; Sudo *et al.*, 1996.

25 HBV is a DNA virus which has a genome consisting of a relaxed, circular, partially duplex DNA species of 3.2 kb. Every nucleotide in the genome is within a coding region, and over half of the sequence is translated in more than one opening reading frame. Several promoters have been identified, driving expression of (a) pre-core proteins, core proteins and polymerase (core promoter); (b) large S surface protein (pre-S1 promoter); (c) medium and small S surface proteins (S promoter); and (d) X protein (X promoter). The core protein encapsulates the viral genome and polymerase, the various S surface 30 proteins make up the protein coat, and the function of the X protein has not been determined.

35 Characterization of the core promoter, which directs the transcription of two greater than genome size messenger transcripts, has been described (for reviews, see Ganem D., in FIELD VIROLOGY 3<sup>rd</sup> Ed. 1996 and Kann M. and Gerlich W., in Viral Hepatitis, 2<sup>nd</sup> Ed.). One of these mRNAs, the pregenomic transcript encodes both the core structural

protein and the viral polymerase as well as template for replication of the negative strand viral DNA. The other 3.5 kb mRNA, the pre-core message, is translated and modified into the soluble viral e antigen. Binding sites for hepatocyte nuclear factors, C/EBP, and Sp1 have previously been described in the core promoter region (as reviewed in Ganem D., in 5 FIELD VIROLOGY, 3<sup>rd</sup> Ed. 1996 and Kann M. and Gerlich W., in VIRAL HEPATITIS, 2<sup>nd</sup> Ed.). The hepatocyte nuclear factors, HNF3 and HNF4, are believed to be important for the liver tropism of HBV. Additional transcription factor binding sites such as C/EBP and Sp1 have been described.

10 A characterization of three HBV promoters is provided herein; a core promoter (SEQ ID NO:16, Fig. 1A), a pre-S1 promoter (SEQ ID NO:22, Fig. 1B), and the HBV-X promoter (SEQ ID NO:25, Fig 3).

The HBV promoters described herein find utility in regulated gene expression which is specific to liver cells.

15 Analysis of the effect of modification of various sequence components of the HBV core, preS1 and X promoters was carried out to identify portions of the promoters that can be used to regulate the expression of a gene operably linked to the HBV core, preS1 or X promoter, respectively, as detailed in Example 3.

20 Luciferase reporter activities of wild type core, X, and preS1 promoter constructs and various modifications thereof were evaluated by transient transfection experiments in cell lines of hepatic origin such as HepG2, Huh7, 22.1.5, and HepAD38.

#### HBV Core Promoter

25 Three regions of interest were identified in the linker scanning analysis of the HBV core promoter. The TATA box, HNF4 (SEQ ID NO:18) and proximal HNF3 (SEQ ID NO:17) sites were identified as the control elements most critical to core promoter activity. As further described in Example 3, three regions of the HBV core promoter, domain 5; domain 8/9 and domain 13 appear to be in the vicinity of cis-elements (HNF-4/HNF-3, HNF-3/Sp1, and the TATA box, respectively) reported in the literature. The results of expression studies presented in Table 9 suggest that domain 8 (SEQ ID NO: 19); domain 30 8/9-1 (SEQ ID NO:20); and domain 13 (SEQ ID NO: 21) are involved in transcriptional activation and that those sequences find utility in regulating the expression of autologous or heterologous genes operably linked to an HBV core promoter comprising SEQ ID NO:20 and/or SEQ ID NO:21:

preS1 Promoter

A luciferase reporter construct was generated using a full-length copy of the HBV genome with the preS1 promoter positioned immediately upstream of the luciferase reporter gene and site-directed mutagenesis was performed to generate four mutants in known transcription factor binding sites and linker scanner mutants. The mutagenized constructs were transiently transfected into Hep3AD38 and tested for promoter activity, as described above. As detailed in Example 3, a known transcription factor binding site, designated HNF1 was found to be critical to preS1 promoter activity.

The results provided herein indicate that the regulatory sequences presented as SEQ ID NO: 23 and SEQ ID NO:24 find utility in regulating the expression of autologous or heterologous genes operably linked to an HBV preS1 promoter comprising one or both of the regulatory sequences.

HBV X Promoter

The HBV X promoter was analyzed by deletion and linker scanning experiments similar to those described for the core promoter.

A luciferase reporter construct was constructed with a full-length copy of the HBV genome and the HBV X promoter positioned immediately upstream of a reporter coding sequence. Promoter constructs were prepared with successive blocks of 21 base pair mutations in the HBV X promoter or known transcription factor binding sites. Mutant constructs were transfected into the hepatoma-derived HepG2 and HepG2 cell lines stably transfected with HBV: 22.1.5 and HepAD38, and the expression of the luciferase reporter gene analyzed to determine HBV promoter activity, as detailed in Example 3. Mutations in domains 3, 4 and 6 as well as double mutants (domains 3 + 6 and domains 4 + 6), yielded the greatest reduction in activity. Additional HBV-X promoter reporter constructs were made with mutations in various known transcription factor binding sites and evaluated for luciferase reporter activity suggesting that domains 18 and 19 are also important for activity of the HBV X promoter.

The results provided herein indicate that the regulatory sequences presented as SEQ ID NO: 26, SEQ ID NO:27 and SEQ ID NO:28 find utility in regulating the expression of autologous or heterologous genes operably linked to an HBV X promoter comprising one or more of the regulatory sequences.

### VIII. Vancomycin-Resistant Enterococci (VRE)

Recently, a rapid increase in the incidence of infection and colonization with vancomycin-resistant enterococci (VRE) has been reported. The observed resistance is of concern due to (1) the lack of effective antimicrobial therapy for VRE infections

- 5 because most VRE are also resistant to drugs previously used to treat such infections, i.e., penicillin and aminoglycosides (CDC, 1993; Handwerger *et al.*, 1993); and (2) the possibility that the vancomycin-resistant genes present in VRE can be transferred to other gram-positive microorganisms.

Although enterococci can be part of the normal flora of the gastrointestinal and  
10 female urogenital tracts, recent studies indicate that enterococci can be transmitted directly in the hospital setting. (See, e.g., Boyce, *et al.*, 1994.) *Enterococci* have been recognized as a cause of nosocomial infection and some strains are resistant to multiple antimicrobial drugs. The most common enterococci-associated nosocomial infections are urinary tract infections, post-surgical infections and bacteremia (Murray, 1990;  
15 Moellering RC Jr., 1992; Schaberg *et al.*, 1991).

Vancomycin has been used extensively to treat *Enterococcus* infection since the late 1970s. Recently, a rapid increase in the incidence of infection and colonization with vancomycin-resistant enterococci (VRE) has been reported.

Resistance to vancomycin and other glycopeptide antibiotics has been  
20 associated with the synthesis of a modified cell-wall precursor, terminating in D-lactate which has a lower affinity for antibiotics such as vancomycin.

Typically gram positive bacterial cell wall synthesis involves assembly,  
membrane transport, incorporation into the cell wall and cross linking of a pentapeptide  
precursor molecule as part of the process of peptidoglycan formation. Vancomycin  
25 functions by forming a complex with the peptidyl-D-ala-D-ala precursor, thereby  
inhibiting precursor transport by transglycosylases and incorporation into the  
peptidoglycan, and weakening the bacterial cell wall. Type A high-level vancomycin  
resistance is achieved via an operon that replaces the C-terminal D-ala with D-lac, such  
that vancomycin binding is inhibited (Walsh C, 1999).

30 The operon is controlled by a two component regulatory system that consists of a sensor protein, VanS and a cytoplasmic response regulator, VanR.

VanS is a two domain transmembrane signaling kinase which undergoes an autophosphorylation at histidine residue (H164). Phospho-VanS in the presence of ATP can undergo phosphotransfer to an aspartate residue on VanR (2). Studies have shown  
35 that phospho-VanR binds with high efficiency to P<sub>vanH</sub> and enhances transcription of the

genes necessary for vancomycin resistance (Haldimann *et al.*, 1997; Holman *et al.*, 1994).

The polysystronic message that gives rise to the genes necessary for high level inducible vancomycin and teichoplanin resistance consists of vanH, A, X, Y and Z. The 5 vancomycin resistance enzyme VanH is an alpha-ketoacid dehydrogenase that stereospecifically reduces pyruvate to D-lactate, which forms an integral part of the bacterial cell wall replacing the vancomycin target dipeptide D-alanine-D-alanine (Stoll *et al.*, 1998; Marshall *et al.*, 1999).

The present invention hypothesizes that a DNA binding molecule properly placed 10 within the vanH promoter will displace phospho-VanR and shut down transcription of inducible resistance genes, thus rendering the bacteria once again sensitive to vancomycin. Although the mechanism is not part of the invention, shutting down transcription of the resistance genes is preferable to shutting down the two component regulatory system due to potential cross talk between P<sub>vanH</sub> and the response regulators 15 of other two component regulatory systems (Silva *et al.*, 1998).

It follows that regulating the expression of the vanH promoter has application to treatment of infectious disease.

DNA sequencing of the vanH promoter region from nine A type strains of VRE 20 showed a high degree of sequence identity with the published A type regulatory region (GenBank Accession N. M97297). A modified pAM401 plasmid (ATCC) was designed containing a VRE promoter sequence upstream of the luciferase gene in a background allowing for growth and maintenance in *E. coli* and *Enterococcus* species. (See Example 4.)

Site directed mutagenesis of the VRE promoter region was carried out by 25 systematically altering short 8 to 10 bp regions of the consensus promoter sequence, including the -35 consensus binding site within the phosphorylated VanR footprint (Arthur *et al.*, 1992). In addition, 20 linker scanning mutants designated M2-M21 were generated and nucleic acid constructs containing the VRE promoter sequence upstream of the luciferase gene were subcloned into the a pRLUC parent vector and transformed into *E. coli*. 30

Each linker scanner promoter mutant was tested for activity, with both up- and down-regulation observed. The largest decrease was to 0% of wild type activity and the 35 largest increase was 1737% of wild type activity. All mutants in the region reported to be footprinted by phosphorylated VanR (M2-M8) showed decreased activity. Increased activity was observed in mutants spanning -30 to +20, suggesting the possibility of a

repressor binding site in this region. Mutants M8 and M9 were shown to consistently result in the highest luciferase activity of the mutants tested (Example 4).

The results provided herein indicate that the regulatory sequences presented as SEQ ID NO: 32, SEQ ID NO:33 and SEQ ID NO:34 find utility in regulating the expression 5 of autologous or heterologous genes operably linked to a VRE promoter comprising one or more of the regulatory sequences.

#### IX. Androgen receptor

Prostate cancer is the most frequently diagnosed cancer in males in the United 10 States. The cancer is treatable if diagnosed early, however, once the cancer metastasizes virtually all patients die within 12-18 months. Current treatments for metastatic prostate cancer involve targeting the androgen receptor (AR) using surgical or chemical means. The androgen receptor (AR) directly regulates gene expression when bound to androgens such as testosterone or dihydrotosterone (DHT) and is required for 15 prostate maintenance. Once androgens are removed, genes regulated by the AR in the prostate are turned on or off resulting in programmed cell death or apoptosis.

The androgen receptor promoter from nucleotides -6000 to +1100 was cloned 20 from genomic DNA by PCR using GenBank sequences for primer design. The amplified promoter sequence was subcloned into pGL3 basic (Promega) for subsequent transient transfection and evaluation of luciferase expression. A large series of deletion constructs were made and tested following transient transfection, as detailed in Example 5.

The results of luciferase expression assays in the AR+ cell line, LNCaP, following 25 transient transfection of various deletion constructs indicate the presence of a repressor binding site between nucleotides -2000 and -200 and multiple activator sites at nucleotides -150 to -100 (homopurine stretch), -100 to -50 (SP1 site), and -50 to +1 (helix loop helix binding site).

More specifically, the results suggest that: (1) the 5' region of the homopurine 30 region represents all of the activity from -150 to -100; (2) the region 3' of the downstream helix-loop-helix sequence contains another 2-fold of activity; and (3) the downstream helix-turn-helix site contains 1.5-fold activity.

The present invention represents the identification of regulatory sequences of the AR promoter, examples of which are presented as SEQ ID NO: 64, SEQ ID NO:65 and SEQ ID NO:66, respectively. The regulatory sequences presented as SEQ ID NO: 64, SEQ ID NO:65 and SEQ ID NO:66 find utility in regulating the expression of autologous or

heterologous genes operably linked to an AR promoter comprising one or more of the regulatory sequences.

X. Her2

5        Her2 (human epidermal growth factor receptor2; c-erbB2, neu) is a tyrosine kinase growth factor receptor implicated in the metastatic growth of a subclass of breast cancers. Her2 overexpression occurs in up to 30% of patients with breast cancer and is associated with an especially aggressive form of the disease characterized by more rapid disease progression and shortened survival. Her2 can be overexpressed in breast  
10      cancer cells, ovarian cancer cells as well as a variety of other cancer cells. Accordingly, regulated expression of Her-2 would be useful to modulating such overexpression.

A 2000-bp fragment of the human Her2 promoter was PCR amplified from genomic DNA using the following oligonucleotides. This purified fragment was subcloned into the vector pGL3-basic (Promega) at the Ncol and HindIII sites for use in  
15      transient transfection luciferase expression assays in the breast carcinoma cell lines MCF-7 (low Her2 expression) and 2R75-1 (high Her2 expression).

The results of studies detailed in Example 6 indicate that the critical regulatory sites for the Her2 promoter lie between nucleotides -125 and -50. More specifically, sequences of interest for regulated expression of Her2 are a repressor sequence (SEQ ID NO:70) downstream of the putative TATA box from -23 to -19; a complex regulatory region (SEQ ID NO:71) which includes both an activator and a repressor component; and a putative TATA box/ets site (SEQ ID NO:72).  
20

The regulatory sequences presented herein as SEQ ID NO:70, SEQ ID NO:71 and SEQ ID NO:72 find utility in regulating the expression of autologous or heterologous genes operably linked to a Her2 promoter comprising one or more of the regulatory sequences.  
25

XI. Beta-lactamase (Bla) promoter

30        The extensive use of beta-lactam antibiotics has resulted in significant bacterial resistance to such treatment. This resistance is generally mediated by lactamases in both gram-positive and gram-negative bacteria. More specifically, the beta-lactamase gene confers ampicillin resistance to a number of types of bacteria including *E. coli*. Recently, therapeutic approaches directed to overcoming such antibiotic resistance have been developed which include the delivery of a beta-lactam antibiotic in combination with a  
35      beta-lactamase inhibitor.

Regulated expression of the beta lactamase gene provides another means to modify such antibiotic resistance. In order to determine which regions of the beta lactamase gene may be used to regulate beta lactamase expression, luciferase reporter constructs were prepared containing a beta-lactamase promoter sequence upstream of 5 the luciferase gene.

Promoter mutants of the natural beta lactamase P3 bla promoter were generated by systematically altering the base pairs of the entire bla promoter sequence (from nucleotides -101 to +43).

Luciferase activities were measured in lysates prepared from *E. coli* XL1 Blue 10 replicates. Mutants which exhibited significantly decreased luciferase activity included those with mutations in the -35 region (-41 to -30, M6); the -10 region (-17 to -6, M8); the start site (-5 to +7, M9); and +20 to +31 (M11). The luciferase activities of these constructs were reduced to 24%, 29%, 15% and 2% of wild type, respectively, as further described in Example 7.

15 Bla promoter linker scanner mutant constructs were generated by introducing 6 or 12 base pair mutations at different locations of entire bla promoter. Renilla luciferase reporter activities of the mutants measured and compared to the activity of the wild type pBla-Renilla luciferase construct.

The regulatory sequences presented herein as SEQ ID NO:77 and SEQ ID NO:78 20 find utility in regulating the expression of autologous or heterologous genes operably linked to a beta lactamase (bla) promoter comprising one or more of the regulatory sequences.

## XII. Utility/Applications

25 The present invention is directed to isolation of various promoters, characterization of the promoters, and in particular characterization of regulatory elements of the promoters. The promoters described herein find utility in regulated gene expression and may function by interaction with natural cellular factors (e.g., transcriptional regulatory proteins) or by interaction with exogenously provided cellular factors or compounds.

30 The promoter may be a minimal or full length promoter. It will be understood that the promoter sequences described herein include minimal promoter elements alone or together with control sequences (also termed "transcriptional and translational regulatory sequences"), involved in expression of a given gene product. In general, transcriptional and translational regulatory sequences include, but are not limited to, the promoter 35 sequence itself, the DNA response element for a transcriptional regulatory protein,

ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences.

The binding of a transcriptional regulatory protein to its corresponding DNA response element serves to regulate the expression of a gene under the control of a promoter operably linked to the promoter. Identification of sequences critical to such binding and regulation provides a framework for controlling the transcription and therefore the expression of a gene under the control of the promoter.

Accordingly, the promoter regulatory sequences described herein may be used to regulate the expression of genes operably linked to the relevant promoter. Such promoter regulatory sequences find utility in the design and construction of heterologous nucleic acid constructs and in the regulated expression of native genes.

The promoter regulatory sequences described herein may also be used in conjunction with a DNA binding compound to regulate the expression of a gene operably linked to the promoter.

In some cases, a given promoter may be regulated by a native factor, for example, the expression of a gene operably linked to a cell type-specific, developmentally regulated, or disease-specific promoter which promotes gene expression in certain tissues without affecting expression in other tissues may be regulated using the sequences described herein.

More specifically, the ability to regulate the expression of genes under the control of a cyclin D1 promoter has application to treatment of various cancers, including, but not limited to, breast cancers, colon cancers and pancreatic cancers.

Interaction between CD40 and CD40L is necessary for B cell activation and isotype switching. Therefore, regulation of the activity of the CD40L gene promoter finds utility in the treatment of various immunological disorders, such as autoimmune disease.

Regulated expression of genes under the control of the HBV-specific core, pre-S and X promoters find utility in the therapy of HBV disease and in the regulated expression of liver cell-specific genes.

Resistance to the antibiotic, vancomycin, which is used to treat *Enterococcus* infection has been associated with the vancomycin resistance enzyme VanH. Therefore, regulated expression of the vanH gene promoter has utility in treatment of *Enterococcus* infection.

Given that the androgen receptor (AR) is currently the target of numerous therapeutic strategies for treatment of prostate cancer, regulated expression of the androgen receptor gene promoter finds utility in the treatment of prostate cancer.

Her2 is a tyrosine kinase growth factor receptor implicated in the metastatic growth of a subclass of breast cancers as well as various other types of cancers. Therefore, regulated expression of the Her2 gene promoter has utility to treatment of cancer.

5       The  $\beta$ -lactamase gene confers ampicillin resistance to *E. coli*. Accordingly, regulated expression of the  $\beta$ -lactamase gene promoter is relevant to modulation of such antibiotic resistance.

10      The sequence information and functional characterization of the promoter regulatory sequences described herein can therefore be used to regulate the transcription of endogenous genes and transgenes (autologous and heterologous genes, respectively), in a variety of useful applications.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

15

#### Material and Methods

Luciferase Assays Cells were washed once with PBS buffer, harvested in 1ml PBS, pelleted, and lysed with 100  $\mu$ l passive lysis buffer (Promega) at room temperature for 15-20 minutes. The cell lysates were centrifuged for 5 minutes, then 10  $\mu$ l of lysate is added to 100 ml of luciferase assay reagent (Promega). Assays were carried out in a luminometer (EG&G Berthold). Luciferase activity is expressed as a rate of light units. Correction for transfection efficiency and variations in harvesting were done by cotransfected an SV40 renilla-luciferase reporter gene (PRL-SV40) or a promoterless renilla-luciferase gene (PRL-Null) and determining the activity of the renilla luciferase internal control in the same Dual Luciferase assay (Promega). After standardization with renilla luciferase activity, a relative luciferase activity was obtained, and the mean and standard deviation from triplicate wells were calculated. In general, transfactions were repeated and reproduced in at least two independent experiments.

30

#### EXAMPLE 1

##### Cyclin D1 promoter analysis

The full-length human cyclin D1 promoter from -1745 to +155 (Fig. 4, SEQ ID NO:1) was PCR amplified and cloned into the firefly luciferase reporter plasmid pGL3 basic. A series of cyclin D1 5' promoter deletions were similarly constructed and cloned

into pGL3-basic. Mutant promoter constructs were assayed in MCF7 cells, a second cyclin D1 overexpressing breast carcinoma cell line, ZR75; a breast cell line that expresses cyclin D1 normally, HMEC; a cyclin D1 overexpressing colon cancer cell line, HCT116; and a cyclin D1 overexpressing pancreatic cancer cell line, PANC-1.

5

Construction of Plasmids

A 1900-bp fragment of the human cyclin D1 promoter was PCR amplified from genomic DNA using the following oligonucleotides: 5'-GCA CGC GTG CTA GCC AGC TGG GCC CTT GT-3' (SEQ ID NO:2) and 5'-ATC CAT GGA AGC TTT GGG GCT CTT 10 CCT GGG CA-3' (SEQ ID NO:3). This purified fragment (SEQ ID NO:1), representing nucleotides -1745 to +155 relative to the transcription start site of the cyclin D1 promoter, was subcloned into the vector pGL3-basic (Promega) at the MluI and HindIII sites to form the reporter -1745D1/LUC. A series of 5' deletions were cloned using polymerase chain reaction of the native promoter plasmid as follows: a 5' deletion to -1590, a 5' deletion to -1440, a 5' deletion to -690, a 5' deletion to -545, a 5' deletion to -390, a 5' deletion to -245, 15 and a 5' deletion to -90, using the PCR primer having the sequence presented as 5'-GCA CGC GTG CTA GCT GGA GCC TCC AGA GGG CTG T-3' (SEQ ID NO:4).

Promoter activities for the 5' deletion constructs were compared to that of the full-length (-1745) cyclin D1 promoter following transfection into asynchronous MCF7 human 20 breast carcinoma cells, which overexpress cyclin D1. Deletion of cyclin D1 promoter regions between -1745 and -245, in the context of the full length promoter (-1745) had little effect on basal promoter activity in MCF7 cells.

Site-directed mutagenesis of the AP1, CRE, E2F, SP1 and Oct1 sites, and linker-scanning mutagenesis of the proximal promoter were generated using the QuickChange 25 mutagenesis system and the parent -1745D1/LUC plasmid. Restriction enzyme analysis and DNA sequencing confirmed the integrity of these constructs.

Mutation of the E2F site {Motokura & Arnold, 1993} resulted in a construct which retained 63% of wild-type activity. Mutation of the CRE element resulted in a construct that retained 32% of wild-type activity, indicating that it is important to basal cyclin D1 30 expression in MCF7 cells..

-60

-37

AACAAACAGTAACGTACACACGGACT  
TTGTTGTCATTGCAGTGTGCCTGA

35

CRE

In addition to MCF7 cells, mutant promoter constructs were assayed in another cyclin D1 overexpressing breast carcinoma cell line, ZR75; in a breast cell line that expresses cyclin D1 normally, HMEC; in a cyclin D1 overexpressing colon cancer cell line, HCT116; and an overexpressing pancreatic cancer cell line, PANC-1. The -1745 wild-type, the -10 deletion or various site-directed mutants of the cyclin D1 promoter were inserted into the promoter-less firefly luciferase plasmid (pGL3-basic) and co-transfected into various cells together with an SV40 promoter driven Renilla luciferase control plasmid. Firefly luciferase activity for each construct was normalized to Renilla luciferase activity and is shown relative to that of the full-length wild-type promoter (-1745).

10

Tissue culture

The human breast carcinoma cell lines MCF7 and ZR75 were maintained in DMEM/F12 medium with 10% fetal bovine serum, 10 µg/ml bovine insulin and antibiotics (penicillin/streptomycin). The human colon carcinoma cell line HCT116 was maintained in McCoy's medium with 10% fetal bovine serum and pen/strep. The human pancreatic cell line PANC-1 was maintained in DMEM/F12 with 10% fetal bovine serum and pen/strep. Human mammary epithelial cells (HMEC) were maintained in Epithelial Growth Media supplemented with bovine pituitary extract (50 µg/ml), hydrocortisone (500ng/ml), hEGF (10ng/ml), and insulin (5 µg/ml). All lines were maintained at 37°C, 5% CO<sub>2</sub>. MCF7, ZR75, HCT116 and PANC-1 cells were purchased from the American Type Culture Collection. HMEC cells were purchased from Clonetics Corp.

25

Transient transfections

30

Cells were transiently transfected with LipofectAMINE (GIBCO Life Sciences) in triplicate in 6-well tissue culture plates (Corning, NY). Equal numbers of cells (3 x 10<sup>5</sup>/well) were seeded in each well 24 hours prior to transfection. Prior to transfection, cells were equilibrated in 800 µl fresh medium (OptiMEM with 5% FBS and pen/strep). Cells were transfected with 5 µg of reporter plasmid containing various different cyclin D1 promoter constructs in 200 µl transfection buffer. After 4 hours incubation with the transfection solution, cells were fed with 4 ml OptiMEM with 5% FBS and pen/strep. Cells were harvested 48 hours after transfection.

35

Analysis of Cyclin D1 promoter elements

Tables 1 and 2, below, show a summary of the results of deletion analysis studies of the cyclin D1 promoter in MCF7 cells. Various 5' deletions or site-directed mutations of

the cyclin D1 promoter were inserted into the promoterless firefly luciferase plasmid (pGL3-basic) and co-transfected into MCF7 cells human breast carcinoma cells, which overexpress cyclin D1 (Buckley, 1993), together with an SV40 promoter driven Renilla luciferase control plasmid. The length of each construct is indicated relative to the transcriptional start site (+1). Firefly luciferase activity for each construct was normalized to Renilla luciferase activity and is shown relative to that of the full-length wild-type promoter (-1745). The data are presented as the mean +/- SEM with a minimum of two independent transfections done in triplicate. Deletion of cyclin D1 promoter regions between -1745 and -245, in the context of the full length promoter (-1745), had little effect on basal promoter activity in MCF7 cells, even though several potential transcription factor binding sites have been previously identified in that region.

Cyclin D1 promoter constructs containing a mutation of the CRE in combination with a mutation of the -30 to -21 region resulting in severely compromised promoter activity in all of the cell lines tested. *In vivo* footprinting experiments carried out as described below demonstrate factor binding at both the CRE and the -30 sites in HCT116 cells.

In all cell lines tested, mutation of the CRE in constructs designated as CREbam and CRE4C5G reduced basal promoter activity considerably although the strongest effect was seen in MCF7 cells. The corresponding wild type sequence is presented as SEQ ID NO:7.

Mutation of the -30 to -21 site reduced basal cyclin D1 promoter activity in some cell lines, but not others. However, in all cell lines tested, mutation of the -30 to -21 site in combination with mutation of the CRE (construct CRE4C-30-21) reduced basal promoter activity considerably and to a greater extent than did mutation of either site alone (Table 1). This suggests that both the CRE and the -30 to -21 sites are involved in transcriptional regulation of basal cyclin D1 promoter activity in all of the overexpressing cancer cell lines tested, as well as in HMEC cells which express normal levels of cyclin D1. The effects of mutations in various other regions of the cyclin D1 promoter are summarized in Table 2, below.

Table 1. Reporter Activity of Cyclin D1 Promoter Constructs

Promoter construct	MCF7 cells (% wild-type)	HCT116 cells (% wild-type)	ZR75 cells (% wild-type)	PANC-1 cells (% wild-type)	HMEC cells (% wild-type)
-1745,(wild-type)	100	100	100	100	100
-10	11 +/- 0.7	22 +/- 1.6	21 +/- 1.1	45 +/- 1.9	50 +/- 4.8
CREbam	32 +/- 1.7	46 +/- 3.3	64 +/- 6.8	52 +/- 7.5	50 +/- 2.1
3'CREm	102 +/- 7.1	86 +/- 8.8	92 +/- 6.4	89 +/- 4.3	74 +/- 2.1
5'CREm	160 +/- 3.6	120 +/- 15.6	N/D	99 +/- 6.3	N/D
CRE4C5G	33 +/- 5.0	69 +/- 5.1	54 +/- 8.3	52 +/- 4.9	N/D
-30 to -21	33 +/- 2.2	91 +/- 12.2	77 +/- 7.0	46 +/- 4.8	78 +/- 4.8
+1 to +9	37 +/- 4.0	46 +/- 4.1	92 +/- 12.5	53 +/- 8.3	74 +/- 5.0
CRE4C/-30 to -2	11 +/- 1.3	30 +/- 4.5	38 +/- 11.4	40 +/- 6.9	26 +/- 1.7
CRE4C/+1 to +9	14 +/- 0.8	32 +/- 4.2	43 +/- 4.0	17 +/- 3.4	N/D

Table 2. Reporter Activity of Cyclin D1 Promoter Constructs

5

5' deletion	% WT activity
-1590	92 +/- 0
-1440	96 +/- 13.1
-690	79 +/- 3.5
-545	82 +/- 2.4
-390	81 +/- 7.1
-310	89 +/- 4.4
-245	78 +/- 7.6
-90	39 +/- 1.2
-10	11 +/- 0.7

Site-directed mutagenesis of the AP1, CRE, E2F, SP1 and Oct1 sites, and linker-scanning mutagenesis of the proximal promoter was carried out to determine the effect on promoter activity. The results indicate that mutation of the E2F site (Motokura et al., 10 1993) resulted in a moderate decrease in activity, while mutation of the CRE element indicated that it is important to basal cyclin D1 expression in MCF7 cells.

A thorough analysis of the CCND1 promoter indicate that the CRE site at -52 is a critical site for cyclin D1 expression in HCT116 colon cancer cells, PANC-1 pancreatic cancer cells, MCF7 and ZR75 breast cancer cells and HMEC breast cells which express cyclin D1 normally. As shown in cyclin D1 Tables 3 and 4 below, mutation of bases -30 to 15 -21 reduced basal promoter activity to 33% revealing another important and novel activator site for cyclin D1 expression in MCF7 cells. Mutation of bases +1 to +9 or +10 to +19 also reduced basal promoter activity, to 37% and 62%, respectively. A double mutant containing mutations in the CRE (SEQ ID NO:7) and the -30 to -21 site (SEQ ID NO:5) 20 was constructed and transfected into MCF7 cells, resulted in only 11% of the activity of

the full-length wild-type promoter retained in all cell lines tested. A double mutant of the CRE in combination with the +1 to +9 site (SEQ ID NO:8) reduced activity to 14%.

Table 3. Reporter Activity of Cyclin D1 Promoter Constructs

5

Mutant Construct	Mutant Sequence	Wild Type Sequence	% Wild Type Activity
mAP1	AAAAAAAATACGCGTGAATGGA	AAAAAAAATGAGTCAGAATGGA	111 +/- 12.8
mAP1ds	TCACCAGTTCTTGGACTGT	TCAGAACGGAGATCACTGT	79 +/- 8.4
mE2F	GGAATTGGATCCCATT	GGAATTTCGGGCATT	63 +/- 10.5
mOCT1	GGGGCGGGATCCTTCT	GGGGCGATTGCTTCT	92 +/- 7.7
mSP1	TGCGCTTTAATTAAAACCT	TGCGCCCGCCCCGCC	105 +/- 5.6
CREbam	CAGTGGATCCACACGG	CAGTAACGTCACACGG	32 +/- 1.7
CRE4C	CAGTAAGGTACACGG	CAGTAACGTCACACGG	33 +/- 5.0
CRE4C5G	CAGTAAGCTCACACGG	CAGTAACGTCACACGG	33 +/- 5.0

Table 4 Reporter Activity of Cyclin D1 Promoter Constructs

Construct	Mutations in -30-21 region	% Wild Type Activity
WT/-1745	GAGTTTTGTT	100
-30 -21/-1745	TCTGGGATCC	33 +/- 2.2
-30 -26/-1745	TCTGGTTGTT	43 +/- 3.5
-25 -21/-1745	GAGTTGGCGG	34 +/- 4.7
-30 -28/-1745	TCTTTTTGTT	33 +/- 6.3
-28 -23/-1745	GATGGGATT	46 +/- 5.1
-23 -21/-1745	GAGTTTTTICC	138 +/- 16.4
10 bp 21x/-1745	GAGTTTTTTAAAG	87 +/- 11.4
8 bp 21x/-1745	GAGTTTTAAAGAG	85 +/- 7.8

10 To examine the proximal promoter region in more detail, a series of site-specific mutations were made in 10 bp segments from -62 to +20, in the context of the full length promoter (-1745) in pGL3 basic. Luciferase activity was evaluated following transfection into MCF7 cells. The results as shown in Table 5 indicate that mutation of either the 10 bp immediately 5' of the CRE (construct 5'CREm), or of bases -20 to -11 increased  
 15 promoter activity suggesting the presence of negative transcriptional regulatory sites in these regions. Site-directed mutagenesis of the -30 to -21 promoter region was carried out and constructs assayed in MCF7 cells. The assay results indicate that bases between -30 and -24 (GAGTTTT, nucleotides SEQ ID NO:6) are the most important for transcriptional activation from this site.

Table 5. Reporter Activity of Cyclin D1 Promoter Constructs

Proximal promoter mutant	% wild type activity
5'CREm	160 +/- 3.6
CRE4C	33 +/- 1.8
3'CREm	102 +/- 7.1
-40 -31	113 +/- 6.8
-30 -21	33 +/- 2.2
-20 -11	165 +/- 19.1
-10 -1	111 +/- 8.8
+1 +9	37 +/- 4.0
CRE4C/-30-21	11 +/- 1.3
CRE4C/+1+9	14 +/- 0.8

The identification of sequences important for transcriptional activation suggest  
5 that it is possible to specifically regulate endogenous cyclin D1 expression in tumor cells  
using a regulatory sequence of the promoter.

***In vivo footprinting*** *In vivo* footprinting of the cyclin D1 promoter was carried out as described in Mueller PR and Wold B, Science, 246(4931):780-786, 1989. Transcription  
10 factor binding at the CRE and in the -30 to -21 region were evaluated by *in vivo* footprinting using dimethyl sulfate (DMS) or UV-light in HCT116 cells. The results of these studies indicate that the CRE is protected in both serum-starved and serum-stimulated cells, consistent with the mode of action for the CRE-binding protein CREB.  
15 (See, e.g., KWOK, 1994.) The results also indicate that a protein is binding to the -30 to -21 region in HCT116 cells and that the site is protected in both serum-starved and serum-stimulated cells. The identity of the factor responsible for binding in the -30 to -21 region remains to be determined.

#### EXAMPLE 2

20        Identification Of CD40 Ligand (CD40L) Promoter Elements

The full-length human CD40L promoter from -1860 to +49 (SEQ ID NO: 9) was PCR amplified and cloned into the firefly luciferase reporter plasmid pGL3-basic. A 1920 bp element of the CD40L promoter region (from -1860 to +49, Figs. 5A-C) was PCR-amplified from genomic DNA (Clontech), using the following primers with 5' Xhol and 3'  
25 HindIII sites to facilitate subcloning.

TTA TGA TAC CTC GAG GGG AGA GCA TTC AGG AAG ATG    (SEQ ID NO:10); and  
TGA ATC ACG AAG CTT TGG TAT CTT CTG GCA GAG AAG    (SEQ ID NO:11).

All 5' deletions were generated in the same manner using primers containing a 3'  
30 HindIII and unique 5' Xhol sequence. Internal deletion and site directed mutants were

generated using Quick Change Mutagenesis (Stratagene) as per the manufacturer's recommendations. Mutant constructs were pre-screened by restriction digest of newly modified sites, and confirmed by sequencing. Mutant constructs were purified using a Qiagen endotoxin free isolation system.

5

Preparation of PBMC Peripheral blood mononuclear cells (PBMC) were purified from buffy coats by Ficoll-Hypaque centrifugation, washed 3 times in Dulbecco's phosphate buffered saline without calcium and magnesium, resuspended at  $5 \times 10^6$  cells/ml in RPMI 1640 media (Gibco BRL), 15% FCS (Gibco BRL) and supplemented with

10 2mM L-glutamine (Gibco BRL), 1x Penicillin/Streptomycin (Gibco BRL) and 10% IL-2 (Hemagen Diagnostics), then plated in 12-well plates at 2mls/well. PBMC were then stimulated with TSST-1 (Toxin Technologies) at a final concentration of 50ng/ml. Cells were cultured at  $3-3.8 \times 10^6$  cells/ml, cultured for one week, then subjected to Ficoll-Hypaque centrifugation, and plated in 12-well plates at 3mls/well at  $3 \times 10^6$  cells/ml.

15 Peripheral blood CD4+ T cells were isolated by depletion with CD8+ magnetic microbeads following the manufacturer's protocol (Milteny Biotec) at week two or week three. Following depletion, the peripheral blood CD4+ T cells were stimulated with irradiated allogeneic whole peripheral blood mononuclear cells and TSST-1. Approximately one week later the cells were stimulated again and transfected twenty hours later.

20 PBMCs were resuspended at  $2 \times 10^7$  cells/ml in complete media and 250 $\mu$ l of the cell suspension transfected with 25  $\mu$ g of a reporter construct and 0.25 $\mu$ g of a co-reporter expressing Renilla luciferase (pRLSV40; Promega) at 250 Volts and 960 microfarads using a Gene Pulser 11 (BioRad). Electroporated cells were plated, allowed to rest for 2 hours at 37°C, then activated with PMA (at a final concentration of 25ng/ml) and 25 ionomycin (at a final concentration of 1.5 $\mu$ M; Sigma). Nine hours post-activation cells were harvested, washed twice in phosphate buffered saline, lysed in 50 $\mu$ l of reporter lysis buffer (Promega) and 20  $\mu$ l of each lysate was assayed for luminescence in an EG&G Berthold Lumat LB9507 luminometer according to the manufacturer's instructions using the Promega's Dual-Luciferase Reporter Assay System.

30

Analysis of CD40L Promoter Elements A series of 5' CD40L promoter deletions were PCR amplified and cloned into the firefly luciferase reporter plasmid pGL3-basic and the authenticity of all clones verified by DNA sequencing. Promoter activities for the 5' deletion constructs were compared to that of the full-length (-1860) CD40L promoter

following transfection into normal expanded T cells and activation with PMA and ionomycin.

Table 6 below, shows the promoter activity of various deletion mutants in activated T cells, some of which affect known transcription factor consensus sites  
5 including potential NF-AT and GATA-3 binding sites, as described by Shimadzu *et al.*,  
1995.

Table 6. Activity of the CD40L promoter with 5' Deletions

5' Deletions	% Wild Type Activity
-1860	100%
to -1220	77%
to -951	91%
to -523	111%
to -280	47% +/- 12%
to -248	25% +/- 6.7
to -160	53%
to -87	45%
to -60	10% +/- 1.1%
to -26	4%

10

The results indicate that (1) deletion of CD40L promoter regions between -1860 and -523 had little or no effect on promoter activity; (2) deletion of the CD40L promoter to -427 resulted in slightly elevated promoter activity suggesting that the region may contain a negative regulatory element; and (3) deletion of the promoter to -280, further to -248,  
15 still further to -60 and still further to -26 reduces activity relative to the wild-type promoter suggesting the presence of activator sites between -427 and -280, between -280 and -248, between -87 and -60, and between -87 and -26.

A series of internal deletions were made within the context of the full-length -1860 promoter in pGL3-basic to address the possibility that deletion of large promoter regions  
20 may remove both positive and negative regulatory elements, and thereby result in cooperative effects. The various deleted CD40L promoter sequences were cloned into the promoter-less firefly luciferase reporter plasmid (pGL3-basic) and co-transfected into expanded T cells together with the SV40 driven Renilla luciferase control plasmid (pRLSV40). Firefly luciferase activity for each construct was normalized to Renilla  
25 luciferase activity and reported relative to that of the full-length promoter (-1860), with the length of each 5' deletion construct indicated relative to the transcriptional start site. All internal deletion clones were verified by DNA sequencing. The internal deletion promoter constructs were then transfected into expanded cultures of T cells and activities were compared to that of the -1860 promoter construct following activation with PMA and

ionomycin. The results presented as the mean +/- standard error of the mean, for a minimum of 2 independent transfections done in triplicate are provided in Table 7, below.

Table 7. Internal Deletions of CD40L and Promoter Activity

Deletion	% Wild Type Activity	Nucleotide Coordinates
-930 to -752	125%	941-1119
-730 to -524	77%	1141-1347
-503 to -428	71%	1368-1443
-406 to -301	36%	1465-1570
-320 to -291	34% +/- 3.5%	1551-1580
-300 to -281	50% +/- 18%	1571-1590
-280 to -231	37%	1591-1640
-230 to -211	15% +/- 3.7%	1641-1660
-230 to -110	13% +/- 2.2	1641-1761
-87 to -68	26% +/- 3%	1784-1803
-160 to -60	20% +/- 6.2%	1711-1811
-72 to -49	4% +/- 1.7%	1799-1822
-61 to -40	2.5% +/- 0.7%	1810-1834
-40 to +9	26%	1831-1880
+9 to +29	14% +/- 2.7	1880-1900

5

Internal deletions which resulted in reduced promoter activity include: (1) the -406 to -301 region, 3-fold reduction in activity relative to wild type; (2) the -320 to -291 region, 3-fold reduction in activity relative to wild type; (3) the -300 to -281 region, 2-fold reduction in activity relative to wild type; (4) the -280 to -231 region, 3-fold reduction in activity relative to wild type; (5) the -230 to -211 region, 6 to 7-fold reduction in activity relative to wild type; (6) the sequence immediately upstream of the -66 NF-AT site (deletion -87 to -68), 4-fold reduction in activity relative to wild type; (7) the -72 to -49 region, 25-fold reduction in activity relative to wild type; (8) the -61 to -40 region, 40-fold reduction in activity relative to wild type; and (9) the +9 to +29 region (downstream of the transcriptional start site), 14% reduction in activity relative to wild type.

In addition, various site-specific mutants constructed within the context of the full-length CD40L promoter (-1860 to +49) in pGL3-basic were co-transfected into normal expanded T cells together with the pRLSV40 control plasmid. Firefly luciferase activity for each construct was normalized to Renilla luciferase activity relative to that of the full-length wild-type promoter (Table 8). In the table, the positions of known transcription factor binding sites are indicated and numbered relative to the transcriptional start site

(+1) with data presented as the mean +/- standard error of the mean for a minimum of 2 independent transfections done in triplicate.

**Table 8 Site Specific Mutations and Promoter Activity**

Mutation	Wild Type Sequence	% Wild Type Activity
-1194 NFkB	GGGATTTCC	83%
-760 NF-AT	TTTTCC	91%
-599 NF-AT	GGAAAA	100% +/- 0%
-306	TTGTCACCTTC	24% +/- 4%
-269 GATA-3	GTGATA	67%
-264 NF-AT	GGAAAA	73% +/- 25%
-66 NF-AT	TTTTCC	32% +/- 4%
-37 to -29 TFIIB	GTGCGCT	53% +/- 19%
-30 to -25 TATA	CTTAAC	47% +/- 12%
-220 to -214	GGCAAG	26% +/- 3.5%
-214 to -208	AATGAA	31% +/- 6.9%
-208 to -202	TATATG	38% +/- 9.9%
-202 to -196	GAAGAA	36% +/- 4
-220 to -208	GGCAAGAATGAA	18% +/- 2.6
-72 to -66	AGCACA	49% +/- 48%
-66 to -60	TTTTCC	31% +/- 5.8
-60 to -54	AGGAAG	42% +/- 2
-54 to -48	TGTGGG	19% +/- 3.8
-48 to -42	CTGCAA	50% +/- 6%
-72 to -60	AGCACATTTC	10% +/- 1.8%
-66 to -54	TTTTCCAGGAAG	7% +/- 1.8%
-66 to -60 and -54 to -48	TTTTCC TGTGGG	14% +/- 2.2%
-66 to -60 and -48 to -42	TTTTCC CTGCAA	15%
-54 to -42	TGTGGCTGCAA	20% +/- 5.5%
-66 to -48	TTTTCCAGGAAGTGTGGG	11% +/- 1.5%
-72 to -60 and -54 to -48	AGCACATTTC TGTGGG	8% +/- 1.4%
-66 to -60 and -54 to -42	TTTTCC TGTGGGCTGCAA	5% +/- 15%

5

The results show that at least 4 regions of the CD40L promoter are critical to expression in activated T cells, as indicated by the levels of luciferase reporter expression and DNA footprinting studies. The regions of the CD40L promoter suggested by these results to be critical to expression in activated T cells include: (1) the site in the vicinity of nucleotide -306 (SEQ ID NO:12), the specific mutation of which results in a 4-fold down regulation of CD40L promoter activity; (2) the region between -230 and -196 (SEQ ID NO:14), based on deletion of the -230 to -211 region (SEQ ID NO:13), which resulted in an 6 to 7-fold downregulation of CD40L promoter activity, and site specific mutations of -220 to -214, -214 to -208, -208 to -202 or -202 to -197, which resulted in a 2.5 to 4-fold down regulation of promoter activity; and (3) the region between -77 and -40 (SEQ ID NO:15), based on the expression level of deletion mutants, wherein an internal deletion of -72 to -49 or -61 to -40 resulted in a 25-fold or 40-fold downregulation respectively. In addition, specific mutations in the composite AP-1/-66 NF-AT site together with a

previously unidentified site located between -48 and -54 indicates a contribution to transcriptional activation through the -48 to -54 site. (See Tables 7 and 8.)

It will be appreciated that some CD40L promoter regions may bind more than one transcription factor, and targeting a DNA-binding compound to the regulatory region of the 5 CD40L promoter, described above, provides a means to inhibit CD40L promoter-mediated transcription through modulation of transcription factor-DNA interactions.

### EXAMPLE 3

#### Hepatitis B (HBV)

10 A linearized unit-length HBV genomic fragment was prepared from an HBV plasmid containing 1.3 copies of a viral genomic sequence such that either the core, the preS1 or the X promoter was at the extreme 3' end. This fragment, when cloned into a reporter construct directionally, placed the promoter element immediately upstream of the reporter coding sequence in order to drive its expression. Luciferase reporter activities of these wild type core, X, and preS1 promoter constructs were evaluated by transient 15 transfection experiments in cell lines of hepatic origin such as HepG2, Huh7, 22.1.5, and HepAD38. Subsequent mutant promoter constructs, prepared by site-directed mutations or linker scanner mutation, were prepared from these wild type clones using mutagenesis methods known in the art.

20

#### HBV Core Promoter

A luciferase reporter construct was constructed with a linearized full-length copy of the HBV genome, with the core promoter positioned immediately upstream and driving the expression of the reporter. Mutagenic primers containing blocks of 15 nucleotides of 25 targeted sequence mutation were designed to generate a series of linker scanner mutant promoter reporter clones using either a *Morph*<sup>TM</sup> (5'Prime to 3'Prime, Boulder, CO) or a QuikChange<sup>TM</sup> (Stratagene, La Jolla, CA) mutagenesis protocol.

Targeted segments of the promoter found to be resistant to mutagenesis were further sub-divided into smaller blocks of mutations consisting of 7-8 nucleotides. This 30 series of linker scanner clones spanned the entire length of the core promoter segment. Mutagenic primers were also used to construct site-directed mutant constructs of known transcription factor binding sites including the hepatocyte nuclear factor sites, HNF3 and HNF4.

To determine potential critical regulatory elements in the core promoter, linker 35 scanner analysis was performed using the series of systemic mutation clones constructed. Each linker scanner mutant construct was evaluated for promoter activity in

transient transfection experiments based on luciferase reporter activity in the hepatoma-derived cell lines HepG2 and HuH7. The HBV stably-transfected cell lines, 22.1.5 and HepAD38, were also used in the linker scanner analysis. An increase or decrease in relative luciferase reporter activity relative to the wild type indicated the presence of potential control elements critical to regulation of gene transcription.

Three regions of interest were identified in the linker scanning analysis. Mutations in domains 5, 8/9, and 13 resulted in 4-10 fold decrease in promoter activity (Table 9). All 3 regions align with cis-elements previously reported in the literature. Domain 5 contains sequences corresponding to a HNF4 transcription factor binding site (AGGACTCTTGGA SEQ ID NO:18). Domains 8/9 contain sequences corresponding to a HNF3 transcription factor binding site (proximal, HNF3-2, GACTGTTGTTT, SEQ ID NO:17). Both of these protein factor sites have been described as important activation elements for the HBV core promoter. Domain 13 mutations abolish the TATA box sequence (CATAAA) of the promoter. A second HNF3 site (HNF3-1, domain 6) has been reported upstream of the one located in domains 8/9. However, mutation of this distal HNF3 site did not show any adverse effects in promoter activity.

**Table 9 Reporter Analysis of Linker Scanner Mutation Clones of the HBV Core Promoter**

Domain	Nucleotide Coordinates <sup>1</sup>	Linker Scanner Sequence	Wild Type Sequence	Percent Wild Type	
1	1601 – 1615	TACATGATATCTTCT	GCACGTGCGCATGGAG	HepG2	HepAD38
2	1616 – 1630	CAAGAATTCCTCAA	ACCACCGTGAACGCC	88	147
3	1631 – 1645	ACAACCCGCGGTAA	CACCAAATATTGCC	79	65
4	1646 – 1660	CCTTGAGGCACGCG	AAGGTCTTACATAAG	28	38
5-1	1661 – 1668	CTCTAGAG	AGGACTCT	34	10
5-2	1668 – 1675	GGTCTAGA	TTGGACTC	22	18
6	1676 – 1690	GACGTCCGTGACCA	TCAGCAATGTCAACG	91	128
7	1691 – 1705	CAATCAAGATCTTAC	ACCGACCTTGAGGCA	76	93
8	1706 – 1720	GCAGGACCCCTCGAG	TACTTCAAAAGACTGT	7	9
9-1	1721 – 1728	GGTGCACC	TTGTTTAA	14	11
9-2	1728 – 1735	CTAGTGT	AAGACTGG	24	17
10	1736 – 1750	TCTTCTAGATTCT	GAGGAGTTGGGGAG	22	22
11	1751 – 1765	TCTCGGCTTGGCCA	GAGATTAGGTTAAAG	24	26
12-1	1766 – 1773	TGCGCATG	GTCTTTGT	103	103
12-2	1771 – 1780	GTGCACCTTC	TGTACTTAGA	37	36
13	1781 – 1795	TTAGTGCTTAAGCCC	GGCTGTAGGCATAAA	16	14
14	1796 – 1810	GCTCGAGTATAAAC	TTGGTCTGCGCACCA	37	68
15	1811 – 1825	TACAACGTACCCGG	GCACCATGCAACTTT	129	185
16	1826 – 1840	GGACAAGCTTAAGC	TTCACCTCTGCCCTAA	229	247

The mutation of several additional regions, as shown in Table 10 showed a reduction in promoter activity of more than 4-fold. These regions, domain 5; domain 8/9 (HNF3 transcription factor binding site); and domain 13 (CATAAA box) appear to align

<sup>1</sup> HBV ayw strain

with the cis-elements (HNF-4/HNF-3, HNF-3/Sp1, and TATA box, respectively) reported in the literature, with the proximal HNF-3 site indicated as one critical element. The results of expression studies presented in Table 9 suggest that domain 8 (SEQ ID NO: 19); domain 8/9-1 (SEQ ID NO:20); and domain 13 (SEQ ID NO: 21) are involved in transcriptional activation.

**Table 10 Reporter Analysis of Site-Directed Mutants of HNF3 and HNF4 Sites of the HBV Core Promoter**

	Nucleotide Coordinates (HBV ayw Strain)	Site-Directed Mutant Sequence	Percent Wild Type HepAD38
Distal HNF3	1680 – 1691	CCAGGGCCCCGA	102
Proximal HNF3	1715 – 1726	GCCGCGGTCTGT	33
HNF4	1661 – 1672	CGTCCGCGGTGA	29

10

Following identification of the TATA box and the HNF4 and proximal HNF3 sites as the control elements most critical for core promoter activity, transcriptional activation as a result of the binding of the TATA binding protein (TBP) and the HNF transcription factors were further studied. It will be appreciated that failure of these protein factors to bind 15 would result in down-regulation of the promoter.

Small DNA-binding compounds were utilized to test their ability to alter the transcription level from wild type and engineered HBV core promoters, either by interference and/or displacement of protein factor binding to its cognate nucleotide binding sequences, as further described in co-owned USSN 09/518,297, filed March 3, 20 2000. The results suggested that a compound binding site may be engineered into a promoter and thereby serve as a means for regulated gene expression of a coding sequence operably linked thereto.

preS1 promoter A luciferase reporter construct was generated containing a full-length copy of the HBV genome with the preS1 promoter positioned immediately 25 upstream of the luciferase reporter gene. Using a wild type luciferase reporter clone, PreSpLuc, as a template, site-directed mutagenesis was performed using a *Morph*™ (5'Prime→3'Prime, Boulder, CO) method to generate four mutants in known transcription factor binding sites and eight 15 bp linker scanner mutants. The mutagenized constructs were transiently transfected into Hep3AD38 and tested for promoter activity, as described 30 above. Table 11 shows the results of the mutation analysis and the ability of the mutated promoters to drive luciferase expression.

Table 11. PreS1 Promoter Activity of Mutants

Construct	Coordinate	Mutated Sequence	% Wild type Activity
HNF1	2720-2732	5' TCGCGAACGGCAG	6
HNF3	2744-2755	5' ACAGCGCGCACA	40
Sp1	2765-2774	5' CGATATCTGC	48
TBP	2778-2784	5' GCGCGCC	34
Domain 1	2702-2716	5' GCGGCGAACACTGCACG	182
Domain 2	2717-2731	5' AGCCGCGGGACGGCA	8
Domain 3	2732-2746	5' GGAACCCAGCTGACA	62
Domain 4	2747-2761	5' GCGCGCACACAGAGC	103
Domain 5	2762-2776	5' GTCTGCAGTTGCGC	115
Domain 6	2777-2791	5' GGCGCGCCTCTCTCC	34
Domain 7	2792-2806	5'CAGCTGACGCTATAA	53
Domain 8	2807-2821	5'GACGGGCCCTTGAG	55

Among known transcription factor binding sites, the HNF1 site appears to be the  
 5 most critical to preS1 promoter activity, as evidenced by the activity of the HNF1 mutant  
 (16-fold reduction in activity). The domain 2 site (SEQ ID NO: 23) overlaps the HNF1 site  
 and a domain 2 mutant showed a 13-fold reduction in activity. A domain 6 mutant showed  
 a 3-fold reduction in activity suggesting that the domain 6 site (SEQ ID NO: 24) is also  
 involved in transcriptional activation. Mutation of the HNF3, Sp1 and TBP binding sites  
 10 resulted in a 2 to 3-fold reduction in reporter activity. In constructs with double mutations  
 in HNF1 and TBP sites, there was no further reduction in reporter activity. In contrast, in  
 Sp1 double mutants with either HNF3 or TBP, there was a further reduction relative to the  
 reporter activity observed for the constructs with a mutation in HNF3, Sp1, or TBP alone.

To further map the HNF1 site, four serial 4 bp mutants with a 1 bp overlap were  
 15 constructed and tested for promoter activity in luciferase reporter constructs (Table 12).

Table 12. HNF1 Linker-Scanning Mutagenesis

Construct	HNF1 sequence	% Wild type Activity
Wild type	GTTAACATTACT	100
HNF1-A	TCGCATCATTAC	4
HNF1-B	GTTCCGAATTAC	3
HNF1-C	GTTAACACGGAC	4
HNF1-D	GTTAACATGCAG	5

20 A series of point mutations spanning the HNF1 binding site were carried out and  
 the mutants tested for luciferase expression following transient transfection. Four of  
 seven mutants retained 14-42% of wild type activity as shown in Table 13, below.

Table 13. Mutants In The HNF1 Site Of The PreS1 Promoter

Consensus	GTT AAT NAT TAA C	Relative Luciferase Activity (%)		
Wild type	GTT AAT CAT TAC TT	100	100	100
mHNF1	TCG CAG ACG GCA GT	5	5	5
HNF1-4A	GTT GAT CAT TAC TT	-	5	-
HNF1-5A	GTT ACT CAT TAC TT	42	30	-
HNF1-5B	GTT AGT CAT TAC TT	20	-	-
HNF1-6A	GTT AAG CAT TAC TT	-	6	-
HNF1-6B	GTT AAC CAT TAC TT	29	-	-
HNF1-9A	GTT AAT CAG TAC TT	-	3	-
HNF1-9B	GTT AAT CAC TAC TT	14	-	-
HNF1-5A6B	GTT ACC CAT TAC TT	-	-	9
HNF1-5A9B	GTT ACT CAC TAC TT	-	-	4

A fluorescence-based assay for characterization of ligands with DNA binding properties was carried out, the results of which are shown in Figure 2. A Hybridization Stabilization assay (HSA) was carried out using a 5'-fluorescent-labeled ssDNA and a 3'-Dabsyl labeled complementary strand of DNA. The oligonucleotides were designed to remain single stranded at room temperature until the ligand binds and duplexes the two strands resulting in quenching of the fluorescent signal. The direct binding of the ligand can then be unquenched by the presence of a more preferred sequence duplex. If a duplex does not have a preferential site for the particular ligand then the signal remains quenched. Figure 2 shows the results of a study where six different duplexes were tested against a particular ligand using a fluorescence-based assay for characterization of ligands with DNA binding properties. A Hybridization Stabilization assay (HSA) was carried out using a 5'-fluorescent-labeled ssDNA and a 3'-Dabsyl labeled complementary strand of DNA. The oligonucleotides were designed to remain single stranded at room temperature until the ligand binds and duplexes the two strands resulting in quenching of the fluorescent signal. The direct binding of the ligand can then be unquenched by the presence of a more preferred sequence duplex. If a duplex does not have a preferential site for the particular ligand then the signal remains quenched. Figure 2 shows the results of a study where six different duplexes were tested against a particular ligand. In the study, fluorescent and dabsyl labeled oligos at 25nM and 35nM were duplexed with the 21X ligand at 75nM. Various other duplexes were then added from 0 to 600nM to determine the sequence binding preference of the ligand. Reactions were in 225 $\mu$ l of

10mM HEPES pH 7.2, 50mM NaCl, 0.1mM EDTA and equilibrated at room temperature overnight.

The sequence that allows for the greatest fluorescence recovery is considered to be a preferred sequence for the ligand. The observed order of binding preferences for 5 21x was: HNF1-21X > TBP wild type > HNF1 wild type > HNF3 wild type > TBP mutant > HNF1 mutant (Figure 2). These results are consistent with the fact that in both the TBP and HNF1 mutant oligos, the majority of A/T bases were changed to G/C bases.

HBV X promoter

10 The HBV X promoter was analyzed by deletion and linker-scanning experiments similar to those described for the core promoter.

A luciferase reporter construct was constructed with a full-length copy of the HBV genome and the HBV X promoter (Fig. 3) positioned immediately upstream of a reporter coding sequence. Promoter constructs were prepared with successive blocks of 21 base pair mutated sequences from the HBV X promoter or known transcription factor binding 15 sites.

Mutant constructs were transfected into the hepatoma-derived cell-line HepG2, HepG2 and cell lines stably transfected with HBV: 22.1.5 and HepAD38, and the expression of the luciferase reporter gene analyzed to determine HBV promoter activity. As indicated in Table 14, mutations in domains 3, 4 and 6 resulted in 28-51% of wild type 20 activity when tested in 3 different cell lines.

Table 14. Linker Scanning Mutants of X Promoter

Construct	Coordinate	Mutated Sequence	% Wild Type Activity (HepG2)	% Wild Type Activity (2.2.15)	% Wild -Type Activity (HepAD38)
Domain 1	1083-1103	5' CCTACTTCGCGACAGGGAGAT	110(343/103)	172/75	230/100
Domain 2	1104-1124	5' AACCAAGGGCCCTTATGGGAGT	95/98	69	58
Domain 3	1125-1145	5' GTGCCCATCGCGAGTCCAAG	33/38	51	40
Domain 4	1146-1166	5' GCAAAATGGGATATCACCATT	59/36	51	45
Domain 5	1167-1187	5' AACTGCAGTGTAACCTGTGGG	113/105	83	119
Domain 6	1188-1208	5' TACAGATATCAAAACAGTTA	33/40	28	33
Domain 7	1209-1229	5' GTTTTAGGATATCGTTAACG	81/85	71	66
Domain 8	1230-1250	5' ACTATACGGATATCCCAAGGG	41/47	64	47
Domain 9	1251-1271	5' GATTACAAGAGATATCGAACG	48(56/39)	80/49	72/32
Domain 10	1272-1292	5' CAGTATTCCAGAAGATATCAG	51/50	62	70
Domain 11	1293-1313	5' GTGGGGAAGATATCACTTGAG	117/168	124	152
Domain 12	1314-1334	5' TTCTACCCACGGCGATATCAG	128	--	--
Domain 23	1335-1355	5' TCGCCAGAGTCGCGAAGCGA	102/100	110	85

25

The wild type sequence for domains 3 through 6, are as follows:

Domain 3: TGTAACAAATACCTGAACCTT (SEQ ID NO:26)  
 Domain 4: TACCCCGTTGCCGGCAACGG (SEQ ID NO:27)  
 Domain 6: GCTGACGCAACCCCCACTGGC (SEQ ID NO:28)

5 Two double mutants (domains 3 + 6 and domains 4 + 6), yielded a 7 to 9-fold reduction in activity relative to wild type controls when evaluated in the HepAD38 cell line (Table 15).

Table 15. Double Mutants of X Promoter

10

Mutant Clones	% Wild Type Activity
M3 + M4	43
M3 + M6	14
M4 + M6	11

Additional HBV-X promoter reporter constructs were made with mutations in various known transcription factor binding sites (Gustin K et al., *Virology* 193, 653-660, 1993; Guo W et al., *J. Virol.*, 1991; Nakamura I et al., *Virology* 191, 533-540, 1992), and 15 evaluated for luciferase reporter activity. The results of those studies which are presented in Table 16 suggest that the EF-C and E factor binding sites are important to activity of the HBV X promoter.

Table 16. Mutants of transcription factor binding sites of X Promoter

20

Domain	Coordinate	Mutated Sequence	% WT Activity (HepG2)	% WT Activity (2.2.15)	% WT Activity (Hep AD38)
NF1	1100-1119	CTCGCCAAC TTACAAGGCCT	109/109	119	93
2C	1119-1134	TTTCTGTGAAACAAT	97/89	74	56
EF-C	1148-1168	CCCCGTTGCCGGCAACGGCC	46/44	36	28
E	1180-1202	CTGACGCAACCCCC	47/39	53	39
NF1	1209-1229	TGGGGCTTGGTCATGGGCCA	88/95	80	78
NF1	1216-1236	TGGTCATGGGCCATCAGCGC	74/77	110	71
X-PBP	1229-1245	ATCAGCGCATGCGTGGAA	56/61	69	48

Given that all HBV-X promoter reporter constructs contained the entire HBV genome, two additional constructs were made: X enhancer/promoter reporter (XpLuc200, Table 17), and the entire HBV genome without X enhancer/promoter-reporter Xp(-) Luc3000, Table 17), to exclude the possibility that there is read-through from other HBV promoters. The XpLuc200 construct was made by amplifying a clone from each of domain 3, 4, and 18 with the forward and reverse primers (SEQ ID NO:29)

and (SEQ ID NO:30), respectively, followed by cloning into the pGL3-Basic vector. The "Xp(-) Luc3000" construct was made by subjecting the wild type construct XpLuc (29-1-5) to site-directed mutagenesis by *Morph*<sup>TM</sup> method. All "XpLuc200" constructs, 3.6, 4.9, 18.13, and 29-1-5, showed approximately 1.5 to 2 fold promoter activity relative to 5 that of each full-length construct, while the Xp(-) Luc3000 construct (29-1-5(-Xp)) showed no promoter activity. These results support the conclusion that the reporter activity presented in Tables 14 and 16 reflect an effect on the HBV X promoter alone, and is not due to upstream HBV promoters (Sp, preSp or Cp).

10 Table 17. XpLuc200 and Xp(-)Luc3000 Constructs

Construct	Presence of promoters				Proximal promoter of reporter gene
	Cp	PreS1p	Sp	Enh1/Xp	
XpLuc200	-	-	-	+	Xp
Xp(-)Luc3000	+	+	+	-	Sp

EXAMPLE 415 Vancomycin-Resistant Enterococci (VRE)

A modified pAM401 plasmid (ATCC) was designed containing a VRE promoter sequence upstream of the luciferase gene in a background allowing for growth and maintenance in *E. coli* and *Enterococcus* species. The vanH promoter (SEQ ID NO:31) was PCR amplified from VRE strain CSUC4 with NcoI and Sall sites added to it. The 20 pAM401 plasmid was cut using XbaI and Sall and triple ligated to the firefly luciferase gene isolated from pGL3 basic (Promega) by cutting XbaI to NcoI and incorporating the vanH promoter into the construct.

Transformants were screened by restriction analysis following PCR amplification and the resulting plasmids electroporated into L-threonine treated *Enterococcus* strain 25 CSUC-4.

Site directed mutagenesis of the VRE promoter region was carried out by systematically altering short 8 to 10 bp regions of the consensus promoter sequence, including the -35 consensus binding site within the phosphorylated VanR footprint (Arthur et al., 1992).

30 20 linker scanning mutants designated M2-M21 were generated and nucleic acid constructs containing the VRE promoter sequence upstream of the luciferase gene were subcloned into the a pRLUC parent vector and transformed into *E. coli*. Figure 7 presents the sequences of vanH promoter mutants M2-M21, wherein each group of 10 nucleotides

in the original vanH promoter sequence shown in the figure was replaced with the mutant sequence, e.g., in M2 the CCCGGGGGGC sequence was inserted in place of the wild type TAATTTTTA sequence. The position of the mutations and corresponding luciferase activity is shown in Table 18.

5       The luciferase expression of selected promoter mutants was analyzed in 3 clinical strains of *Enterococcus* to ascertain if the effect of modified promoter elements on expression is consistent between strains. CSUC-4, the initial strain assayed, UCD-3 and UL-178 were used in the analysis. The M9 clone consistently resulted in the highest luciferase activity of the mutants tested. Among the other mutants M8 also had a  
10      consistent effect on induction.

(See Table 18.)

Table 18. vanH Promoter Mutants And Reporter Activity

Construct	Coordinate	Mutated Sequence	% Wild Type Activity (UCD3)	% Wild Type Activity (UL17)	% Wild Type Activity (CSUC4)
M2	-100 to -91	CCCGGGGGGC	120.4	53.6	10.7
M3	-90 to -81	TTCGGGGGA	108.7	38.7	10.3
M4	-80 to -71	CCTAGGCGAG	—	—	0.4
M5	-70 to -61	GGCGCGGGGA	—	—	1.6
M6	-60 to -51	GCGCGCCCGG	36.5	10.3	0.4
M7	-50 to -41	CCACGCGCGC	45.5	18.9	1.8
M8	-40 to -31	GCGCGCTCCC	0.1	0.0	1.3
M9	-30 to -21	ATTGGTACCA	152.5	100.9	1202
M10	-20 to -11	GGCGCGCTGC	—	—	32.6
M11	-10 to -1	TCAGCGCGCA	1.3	—	1405
M12	+1 to +10	ATGCGCGCAT	—	—	1737
M13	+11 to +20	TTAACGGGGA	—	—	770.7
M14	+21 to +30	TGGAGCGCGC	—	—	115.2
M15	+31 to +40	TCCGCGCGCT	—	—	50.6
M16	+41 to +50	CACGCGCGCA	—	—	23.6
M17	+51 to +60	ACGGAATTCA	—	—	2.4
M18	+61 to +70	AAAGCGCGCG	—	—	76.3
M19	+71 to +80	GGTACCAAGG	—	—	57.3
M20	+81 to +90	GACAGCTGCT	—	—	0.0
M21	+91 to +100	TTGGTTAACG	—	—	12.6

15       Each linker scanner promoter mutant was tested for activity, with both up- and down-regulation observed. The largest decrease was to 0% of wild type activity and the largest increase was 1737% of wild type activity. All mutants in the region reported to be footprinted by phosphorylated VanR (M2-M8) showed decreased activity. Increased activity was observed in mutants spanning nucleotides -30 to +20 (M9-M13), suggesting  
20      the possibility of a repressor binding site in this region. Of particular interest are putative

activator sequences which correspond to M6 (SEQ ID NO:32) and M8 (SEQ ID NO:33), and a putative repressor sequence which corresponds to M12 (SEQ ID NO:34).

EXAMPLE 5

5           Androgen receptor

The androgen receptor promoter from nucleotides -6000 to +1100 (Figs. 8A-C, SEQ ID NO:35), was cloned from genomic DNA by PCR using GenBank sequences and subcloned into pGL3 basic (Promega) for subsequent transient transfection.

10           A large series of deletion constructs were made and tested in the androgen dependent prostate cell line, LNCaP, following transient transfection.

The deletion constructs were made using the following PCR primer pairs: for the -6000+1 construct (SEQ ID NO:36) and (SEQ ID NO:37); for the -4000+1 construct (SEQ ID NO:38) and (SEQ ID NO:39); for the -2000+1 construct (SEQ ID NO:40) and (SEQ ID NO:41); for the -2000+1100 construct (SEQ ID NO:42) and (SEQ ID NO:43); for the -200+1 construct (SEQ ID NO:44) and (SEQ ID NO:45); for the -200+100 construct (SEQ ID NO:46) and (SEQ ID NO:47); for the -400+1 construct (SEQ ID NO:48) and (SEQ ID NO:49); for the -300+1 construct (SEQ ID NO:50) and (SEQ ID NO:51); for the -150+1 construct (SEQ ID NO:52) and (SEQ ID NO:53); for the -100+1 construct (SEQ ID NO:54) and (SEQ ID NO:55); for the -50+1 construct (SEQ ID NO:56) and (SEQ ID NO:57); for the -200+125 construct (SEQ ID NO:58) and (SEQ ID NO:59); for the -200+71 construct (SEQ ID NO:60) and (SEQ ID NO:61); and for the -200+50 construct (SEQ ID NO:62) and (SEQ ID NO:63).

25           The following deletion constructs were tested for luciferase activity with the results in parentheses presented as % of the -200+1 control: -6000+1 (38%), -4000+1 (31%), -2000+1 (45%), -400+1 (93%), -300+1 (100%), -200+1 (100%), -150+1 (109%), -100+1 (62%), -50+1 (28%), -2000+1100 (100%), -200+1100 (459%), +1+1100 (114%), -200+200 (562%), -200+150 (474%), -200+125 (314%), -200+100 (168%), -200+71 (153%) -200+50 (87%) and basic promoter construct (5%).

30           The results of transient transfection assays in the AR+ cell line LNCaP illustrate a repressor, and multiple activator sites at nucleotides -150 to -100 (homopurine stretch), -100 to -50 (SP1 site), and -50 to +1 (helix loop helix binding site).

35           The results indicate that: (1) the untranslated region (UTR) from +1 to +1100 contains two critical regions for optimal activity, sites between +125 and +100 and between +71 and +50; (2) a repressor site may exist between -2000 and -400; and (3) the activity of the proximal promoter region is derived from sequences between -150 and -100

(approximately 2-fold) and between -100 and -50 (an additional 2-3 fold) and -50 to +1 (an additional 4-5 fold).

- Additional site specific mutants were generated as follows: delta HP, a 40 bp internal deletion of the homopurine stretch, delta HP (5'), delta HP (3'), HLH-us, SPI, 5 HLH-ds, the 3' 10 bp of HLH-ds (HLH-3), the 5' 10 bp of HLH-ds (HLH-5) and a double mutant of delta HP and HLH-ds (delta HP /HLH-ds) all in the context of the 200+1 construct. The results of transient transfection studies in LNCaP cells expressed as % of the -200+1 control are presented in Table 19.

10 Table 19. Luciferase activity of Promoter constructs

Promoter Construct	Luciferase Expression (% of control)
-200+1 (control)	100
delta HP	40
delta HP (5')	41
delta HP (3')	89
HLH-ds	63
delta HP/HLH-ds	40
HLH-us	88
SPI	111
HLH-3	42
HLH-5	136

These results suggest that: (1) the 5' portion of the homopurine region represents all of the activity from -150 to -100; (2) the region 3' of the downstream helix-loop-helix 15 sequence contains another 2-fold of activity; and (3) the downstream helix-turn-helix site contains 1.5-fold activity.

Of particular interest are the HLH-ds and HLH-3 deletion mutants and the 5' HP 20 mutant, which resulted in a significant decrease in luciferase activity indicating the presence of an activator site. The corresponding wild type sequences for these mutants are presented as SEQ ID NO: 64, SEQ ID NO:65 and SEQ ID NO:66, respectively.

#### EXAMPLE 6

##### Her2

25 A 2000-bp fragment of the human Her2 promoter (Fig. 9, SEQ ID NO:67) was PCR amplified from genomic DNA using the following oligonucleotides:  
 5'- GCA CGC GTA AGC TTC AGG CCC CAC AAA ACC TA-3' (SEQ ID NO:68) and  
 5'- CGC TCG AGC CAT GGC TCC GGC TGG ACC CGG CTG GG-3' (SEQ ID NO:69).

This purified fragment was subcloned into the vector pGL3-basic (Promega) at the

Ncol and HindIII sites for use in transient transfection assays in breast carcinoma cell lines MCF-7 (low HER2 expression) and MDA-MB-453 (high HER2 expression).

In addition, several deletion constructs were made in a Her2 luciferase reporter containing a 2 kb promoter fragment cloned into pGL3-Basic. The reporters were 5 transiently transfected into the MCF7 and ZR75 cell lines. Table 20 shows the reporter activity for each promoter construct with the modified sequence portion indicated as underlined. The results indicate that the critical regulatory sites for the Her2 promoter lie between nucleotides -125 and -50.

10 Table 20. Luciferase Reporter Activity of Various Her2 Promoter Constructs in MCF7 and ZR75 Cells.

Construct	Sequence (modification presented as underlined)	% Wild Type Activity (MCF7/ZR75)
Her2 wild type	GAGCTGGGAGCGCGCTTGCTCCCAATCACCGGAGAAGGA	100/100
100 to 85	<u>GATGGATC</u> TATACCGTCCC <u>AATCACCGGAGAAGGA</u>	22/33
80 to 65	GAGCTGGGAGCGCGCTTGCTCCAGGATCCATCACCTGA	30/29
90 to 75	GAGCTGGGAGCG <u>GATGGAT</u> CCAA <u>ACC</u> GAACGGAGAAGGA	9/12
87 to 79	GAGCTGGGAGCGCG <u>GATCCA</u> ATATCACCGGAGAAGGA	16/12
84 to 76	GAGCTGGGAGCGCGCTTGAGGATCCGAACGGAGAAGGA	18/23
84 to 78	GAGCTGGGAGCGCGCTT <u>TAGATCT</u> TACCGGAGAAGGA	/17
81 to 76	GAGCTGGGAGCGCGCTAAGCTTCAATCACCGGAGAAGGA	/23
90 to 82	GAGCTGGGAGCA <u>ATGGAT</u> CCAA <u>ATCACCGGAGAAGGA</u>	505/434
84 to 81	GAGCTGGGAGCGCGCTT <u>TAGACCA</u> ATCACCGGAGAAGGA	306/297
93 to 85	GAGCTGGGAT <u>AGGA</u> TCTCTCC <u>AA</u> TCACCGGAGAAGGA	41/62
81 to 73	GAGCTGGGAGCGCGCTTGCTCA <u>AGGA</u> TCCAGAGGAAGGA	70/71
93 to 88	GAGC <u>GG</u> AT <u>CCCGCG</u> CTTGCTCC <u>AA</u> TCACCGGAGAAGGA	/46
87 to 82	GAGCTGGGAG <u>GGAT</u> C <u>TGCTCC</u> <u>AA</u> TCACCGGAGAAGGA	/72
75 to 70	GAGCTGGGAGCGCGCTTGCT <u>CC</u> AG <u>CTCC</u> GGAGAAGGA	/132
75 to 70	GAGCTGGGAGCGCGCTTGCT <u>CCGG</u> AT <u>CCCC</u> GGAGAAGGA	60/60

To further delineate the critical site(s), a series of linker scanner Her2 luciferase 15 reporter mutants was made from nucleotides -130 through -55. The constructs designated 10085, 9075, 8065, and 7055 (indicating the bases mutated; e.g., 10085 indicates that bases from -100 through -85 were mutated, etc.) were tested in transient transfections in ZR75 and MCF7 cells with the results presented in Table 23 as % activity relative to the wild type promoter.

20 The results clearly implicate the -90 to -75 region as critical to the activity of the Her2 promoter.

Mutations were made in various regions of the Her2 promoter, including an AT-rich 25 region around and including a putative TATA box (TB, "TATAAGA"), a putative TATA box (T5B, CTTGAGGAAGGAATCGAATGAAGTTGT), an AT stretch downstream of the putative TATA box (T3B, CTTGAGGAAGGTATATAATCCGGAAAGTTGT), a putative ets site (EP), a double mutant of the AT-rich region around and including the putative TATA box

(TATA/Ets, CTTCGATCGGATCCGCCGGAAGTTGT), and the putative ets site (TBEP, "GAGGAA") as well as a deletion to -215. Sequence modifications are indicated as underlined.

5 Luciferase reporter constructs were prepared with the various Her2 promoter sequences immediately upstream of the reporter coding sequence. The reporters were transiently transfected into MCF7 and ZR75 cells and the resulting luciferase expression reported as the % of wild type (Table 21).

10 Table 21. Luciferase Reporter Activity of Various Her2 Promoter Constructs

Promoter Construct	% WT Activity MCF7 cells	% WT Activity ZR75 cells
Basic	3.3	2.1
Wild type	100	100
-215	143	195
TB	796	432
T5B(TATA)	64	44
T3B	521	351
EP (ets)	69	62
TBEP	843	449

15 The data suggest that sequences upstream of nucleotide -215 are not critical for regulation. As shown in Table 21, mutating the TATA box or the ets site causes a modest decrease in transcription, suggesting that a repressor site lies just downstream of the TATA box. The sequence near the putative TATA box and putative ets site is shown below.

20 CTGCTTGAGGAAGTATAAGAATGAAGTTGT  
ets            TATA box

An additional deletion construct, -50, was made in a Her2 reporter containing a 2 kb promoter fragment cloned into pGL3-Basic and compared to the -215 deletion. The reporters were transiently transfected into MCF7 and ZR75 cells line. The results indicate that critical regulatory sites for the Her2 promoter lie in the -215 to -50 region.

25 Several additional deletion constructs were made in a Her2 luciferase reporter containing a 2 kb promoter fragment cloned into pGL3-Basic. The reporters were transiently transfected into MCF7 and ZR75 cells line. The results presented in Table 22 (expressed as % wild type luciferase activity), indicate that the region of the Her2 promoter between -125 and -50 contains critical regulatory sites.

Table 22. Luciferase Reporter Activity of HER2 Deletion Constructs

Her2 promoter construct	MCF7 cells	ZR75 cells
Basic	6.1	4.1
Wild type	100	100
deletion of 5' end to -215	215	167
deletion of 5' end to -150	58	41
deletion of 5' end to -125	65	40
deletion of 5' end to -100	27	16
deletion of 5' end to -50	4.8	3.7

Further experiments were carried out to determine if the sequences identified as  
5 important in ZR-75 and MCF-7 cells are also important in other breast cancer cell lines.  
Two cell lines SKBR-3 (SK) and BT-474 (BT), were selected which overexpress Her2 at  
higher levels than do either ZR-75 or MCF-7 cells. A summary of the data from 3  
transient transfections is presented below in Table 23, together with the results of parallel  
studies done in ZR-75 cells (ZR).

10

Table 23. Luciferase Reporter Activity of Her2 Mutants in BT-474 and SKBR-3 Cells

Reporter	Transfection 1			Transfection 2			Transfection 3		
	SK	BT	ZR	SK	BT	SK	BT	ZR	
Her2 WT	100	100	100	100	100	100	100	100	
Basic	0.8	3.2	2.0	0.6	2.8	0.5	3.5	2.1	
9082-1	150	293	416	269	243	177	296	337	
8481-3	295	286	406	159	220	197	251	305	
TATAB-3	446	1014	881	733	961	500	718	608	
8478-10	49	40	19.5	41	52	23	52	27	
CATb-21	27	69	52	25	76	20	72	59	
9075-8		10	30	10	9	39			
-50-3	3	6	4	2	7.5				
-100-3	10	21	21	9	25				
-150-10	19	45	48	16	51				

The comparative results of luciferase expression assays in the 3 cell lines suggest  
15 the following: (1) the Her2 promoter is 4-5 fold stronger in SKBR-3 cells than in BT-474  
cells and 3-4 fold stronger than in ZR75-1 cells; (2) the TATA-Bam mutation results in less  
up-regulation in SKBR-3 cells than in the other two cell lines; (3) the CCAAT box is more  
important in SKBR-3 (4-5 fold decrease) than in either ZR75-1 (2-fold down) or BT-474  
(less than 2-fold down) cells and (4) the CCAAT box may be an appropriate target for  
20 regulation of Her2.

Based on the results provided above, sequences of interest for regulated expression of Her2 are a repressor sequence "GAATGAAGTT" (SEQ ID NO:70) downstream of the putative TATA box at -23 to -19; the complex regulatory region "CGCTTGCTCCCAATC" (SEQ ID NO:71), which has both activator and repressor components and the TATA box/ets site, "GAGGAAGGTATAA" (SEQ ID NO:72), wherein the ets sequence is "GAGGAAG" and the TATA box sequence is "TATAA".

#### EXAMPLE 7

10     Beta-lactamase (Bla) promoter

The natural beta-lactamase promoter P3 (SEQ ID NO:73), shown below, lies near the coding sequence of beta-lactamase (bla), initiating transcription at 35 bases 5' to the ATG translation initiation codon. The P3 promoter contains a Pribnow box (GACAATA) at the -10 region and a -35 consensus sequence, TTCAAA. The -35, -10, start site and 15 ribosome binding site, respectively in the 5' to 3' order, are indicated as underlined, below.

20     GACGTCAGGTGGCACTTTCGGGAAATGTGCGCGAACCCCTATTGTTTATTTT  
      CTAATACATTCAAATATGTATCCGCTCATGAGAACAATAACCCTGATAATGCTTCAATA  
      ATA TTGAAAAAGGAAGAGT (natural beta-lactamase promoter P3, SEQ ID NO:73)

25     Renilla luciferase reporter constructs were prepared in the pACYC177 vector, wherein the wild type beta-lactamase promoter driven Renilla luciferase construct was designated pBla-rluc. The control promoter-less construct was designated pNull-rluc, and the luciferase negative construct designated pBla-bla.

30     Bla promoter mutants (designated "M#") of the natural P3 bla promoter were generated by systematically altering the base pairs of the entire bla promoter sequence (from nucleotides -101 to +43). In general, mutants were generated by introducing 6 to 12 base pair mutations at different locations of entire Bla promoter by Quick Change, by replacing purines with pyrimidines and vice versa and incorporating restriction sites in the 35 sequence.

35     Luciferase activities of various Bla mutants were measured in lysates prepared from *E. coli* XL1 Blue replicates and compared to that of wild type pBla-rluc. Mutants which exhibited significantly decreased luciferase activity include the -35 region (-41 to -30, M6); the -10 region (-17 to -6, M8); the start site (-5 to +7, M9); and +20 to +31 (M11) which exhibited luciferase activities which were reduced to 24%, 29%, 15% and 2% of wild type, respectively, as shown in Table 24, below.

Table 24. Sequences of Bla Promoter Mutants and Luciferase Reporter Activity

Mutant	Location	Wild Type Sequence	Mutated Sequence	Luciferase Activity (% Wild Type)
M6	-41 to -30	AATACATTCAAA	CCGGCCGGACCC	24%
M21	-35 to -30	TTCAAA	GGACCC	28%
M8	-17 to -6	CATGAGACAATA	ACGCGTCACCGC	29%
M30	-8 to -3	TAACC	CGCCAA	24%
M9	-5 to +7	ACCCGTATAAAT	CAAAGTGCACCG	15%
M11	+20 to +31	TTGAAAAAGGAA	GGGCCCTTCC	2%

5 Luciferase activity of mutants M6 (-41 to -30; SEQ ID NO:75) and M21 (-35 to -30) revealed that the -35 region is critical to promoter activity, as indicated by a reduction in luciferase activity to 24% and 28%, respectively. Luciferase activity of mutant M8 (-17 to -6; SEQ ID NO:76) and M30 (-8 to -3) revealed that the -10 pribnow box region is also critical, as indicated by a reduction in luciferase activity to 29% or 24%. Two additional regions important for luciferase activity are the start region (M9; SEQ ID NO:77) and the 10 ribosome binding site region (M11; SEQ ID NO:78), as indicated by a reduction in luciferase activity to 15% and 2%, respectively.

15 The sequence of the -101 to +35 region of a modified BlaMT promoter (SEQ ID NO:74) is presented below with lower case letters indicating mutations relative to the natural P3 Bla promoter sequence.

GACGTCAGGTGGCACTTTGGGGAAATGTGCGCGGAACCCCTATcTGTTgTTCTTc  
TAgAcACATTCAcAcATGTATCCGCTCATGAGACAATAACCCCTGATAATGCTTCAATgA  
cATTGAgAAAGGAAGAGT (modified BlaMT promoter, SEQ ID NO:74)

20 Table 25. Sequences of pBlaMT and Mutant pBlaMT Constructs

Mutants	Sequence(-35 to +7 of BlaMT promoter )
PblaMT	TTCACACATGTATCCGCTCATGAGACAATAACCCCTGATAAAAT
pBlaMT(-35)	<u>TTtAaAtATGTATCCGCTCATGAGACAATAACCC</u> CTGATAAAAT
pBlaMT(-10)	TTCACACATGTATCCGCTCATGAGA <u>AAATA</u> ttCTGATAAAAT
pBlaMT(-10p)	TTCACACATGTATCCGCTCATGAGACAATAACCCCTGATgAAT
pBlaMT(-10/+)	TTCACACATGTATCCGCTCATGAGACAATAAA <u>tTGAcgAAT</u>
pBlaMT (+1)	TTCACACATGTATCCGCTCATGAGACAATAAC <u>tTATAAAAT</u>

25 Table 25 depicts the location and sequence of various mutant BlaMT promoter constructs. Lower case letters indicate the mutations relative to the pBlaMT sequence and underlined sequences indicate the location of potential compound binding sites. As detailed in co-owned PCT Publication No. WO 00/52179, when a sequence immediately downstream of the start site in various pBlaMT mutant constructs [e.g.,

pBlaMT, pBlaMT(+1), pBlaMT(-35) and pBlaMT(-10)] was targeted by a DNA binding compound, the activity of each promoter was up-regulated.

The data presented herein provides an analysis of the regulatory regions of various promoters and shows that once the regulatory region of a promoter is identified, it can be targeted by both cellular factors (native or exogenously provided) and by compounds in order to effect regulated expression of a coding sequence operably linked thereto.

Table 26. Sequence Listing Table

DESCRIPTION	SEQ ID NO
cyclin D1 promoter -1745 to +155 (wild-type, Fig. 4)	1
cyclin D1 primer for PCR amplification of promoter from genomic DNA 5'-GCA CGC GTG CTA GCC AGC TGG GCC GCC CTT GT-3'	2
cyclin D1 primer for PCR amplification of promoter from genomic DNA 5'-ATC CAT GGA AGC TTT GGG GCT CTT CCT GGG CA-3'	3
cyclin D1 primer for PCR cloning of 5' deletions: 5'-GCA CGC GTG CTA GCT GGA GCC TCC AGA GGG CTG T-3'	4
cyclin D1 -30-21 wild type sequence: GAGTTTTGTT	5
cyclin D1 -30-24 wild type sequence: GAGTTTT	6
cyclin D1 CRE wild type sequence: CAGTAACGTACACACGG	7
cyclin D1 (+1 to +9) wild type sequence: CCTCCAGAGG	8
CD40L promoter (human, full-length) -1860 to +49 (Fig. 5A-C)	9
CD40L, D1 primer for PCR amplification of promoter from genomic DNA 5'-TTA TGA TAC CTC GAG GGG AGA GCA TTC AGG AAG ATG-3'	10
CD40L, D1 primer for PCR amplification of promoter from genomic DNA 5'-TGA ATC ACG AAG CTT TGG TAT CTT CTG GCA GAG AAG-3'	11
CD40L site between -320 and -297: GAT GAA TTT GTC ACT TTC CTT GAA	12
CD40L site between -230 and -211: GAC ATT TCA AGG CAA GAA TG	13
CD40L site between -230 and -196: ACA TTT CAA GGC AAG AAT GAA TAT ATG GAA GAA GA	14
CD40L site between -77 and -40: TACGA AGCACATTTCCAGGAAGTGTGGGCTGCAACG	15
HBV core promoter sequence (Fig. 1A)	16
HBV core promoter proximal, HNF3-2 site: GACTGTTTGTT	17
HBV core promoter HNF4 transcription factor binding site: AGGACTCTTGGA	18
HBV core promoter domain 8 wild type sequence: TACTTCAAAGACTGT	19
HBV core promoter domain 8 and 9-1 wild type sequence: TACTTCAAAGACTGTTTGTTAA	20
HBV core promoter domain 13 wild type sequence: GGCTGTAGGCATAAA	21
HBV pre-S1 promoter sequence (Fig. 1B)	22
HBV pre-S1 promoter domain 2 wild type sequence: CTA GTT AAT CAT TAC	23
HBV pre-S1 promoter domain 6 wild type sequence: TTA TAT AAG AGA GAA	24
HBV-X promoter sequence (Fig. 3)	25
HBV-X promoter domain 3 wild type sequence: TGTAAACAATACCTGAAACCTT	26
HBV-X promoter domain 6 wild type sequence GCTGACGCAACCCCCACTGGC	28
Forward primer for construction of XpLuc200 CACCGAAGCTTAAGCAGGCTTCACTTTCTCG	29
Reverse primer for construction of XpLuc200 CAGTACCGGAATGCCAAGCTTCGATG	30

DESCRIPTION	SEQ ID NO
vanH promoter sequence (Fig. 6) GAGATGTATAATTAGGAAAATCTCAAGGTTATCTTACTTTCTTAGG AAATAACAATTAAATTAAGAAACGGCTCGTCTTACACGGTAGACTTAATAC CGTAAGAACGAGCCGTTTCGTTCTCAGAGAAAGATTGACAAGATTACCAT GGCATCCCCGTTTATTTGGTGCCTTCACAGAAAGGGTTGGTCTTAATT	31
vanH wild type promoter sequence corresponding to M6: TTAGGAAATT	32
vanH wild type promoter sequence corresponding to M8: TATTAAGAAA	33
vanH wild type promoter sequence corresponding to M12: CGTAAGAACG	34
Androgen receptor (AR) promoter sequence from -6000 to +1100 (Figs. 8A-C)	35
AR: forward PCR primer for construction of -6000+1 deletion construct CACCGGTGGTACCTCTAGAAAATAATCCCACATTGAATCCC	36
AR: reverse PCR primer for construction of -6000+1 deletion construct AGCTGGCTCCCCGGGATCTCGGAGGGGCGC	37
AR: forward PCR primer for construction of -4000+1 deletion construct CACCGGTGGTACCCAGACAGTGACAGGACTAAACGGGAAAT	38
AR: reverse PCR primer for construction of -4000+1 deletion construct AGCTGGCTCCCCGGGA	39
AR: forward PCR primer for construction of -2000+1 deletion construct CACCGGTGGTACCTATAACATTATGTCTTTAAATGAC	40
AR: reverse PCR primer for construction of -2000+1 deletion construct AGCTGGCTCCCCGGGATCTCGGAGGGGCGC	41
AR: forward PCR primer for construction of -2000+1100 deletion construct CACCGGTGGTACCTATAACATTATGTCTTTAAATGAC	42
AR: reverse PCR primer for construction of -2000+1100 deletion construct CCGCACATGGTGAGCTGGCTGAATCTCCA	43
AR: forward PCR primer for construction of -200+1 deletion construct CCGGGTACCTGCCCTGCCAACGCTGCGCC	44
AR: reverse PCR primer for construction of -200+1 deletion construct AGCTGGCTCCCCGGGATCTCGGAGGGGCGC	45
AR: forward PCR primer for construction of -200+100 deletion construct CCGGGTACCTGCCCTGCCAACGCTGCGCC	46
AR: reverse PCR primer for construction of -200+100 deletion construct AGCTGGCTCCCCGGGATCTCGGAGGGGCGC	47
AR: forward PCR primer for construction of -400+1 deletion construct CAGAACATTCTATCGATAGGTACCGAGCAGGTATTCCTATCGTCTTTCC	48
AR: reverse PCR primer for construction of -400+1 deletion construct GGAAAAGGACGATAGGAATACCTGCTCGGTACCTATCGATAGAGAAATGTTCT G	49
AR: forward PCR primer for construction of -300+1 deletion construct CAGAACATTCTATCGATAGGTACCAAATCTGGAGCCCTGGGCCCTAAACCT	50
AR: reverse PCR primer for construction of -300+1 deletion construct AGGTTAGGCGCCAGGGCTCCAGATTGGTACCTATCGATAGAGAAATGTTCT G	51
AR: forward PCR primer for construction of -150+1 deletion construct CAGAACATTCTATCGATAGGTACCGGCGTTAGCGCGCGTGAGGGGAG	52

DESCRIPTION	SEQ ID NO
AR: forward PCR primer for construction of -100+1 deletion construct CAGAACATTTCTATCGATAGGTACCGGGAAAAGGAGGTGGGAAGGCAAGGAGGCC	
AR: reverse PCR primer for construction of -100+1 deletion construct GGCCTCCTGCCTCCCACCTCCTTCCCGTACCTATCGATAGAGAAATGTTCTG	55
AR: forward PCR primer for construction of -50+1 deletion construct: CAGAACATTTCTATCGATAGGTACCGCTCGCAAACGTGGCATTTGCTCTCCA CCTCCC	56
AR: reverse PCR primer for construction of -50+1 deletion construct: GGGAGGTGGAGAGCAAATGCAACAGTTGCGAGGGTACCTATCGATAGAGAA ATGTTCTG	57
AR: forward PCR primer for construction of -200+125 deletion construct CCAGTGCTGTACAGGAGCCGAAGGGACGCACCCCATGGAAGACGCCAAAAAC ATAAAGAAAGGCC	58
AR: reverse PCR primer for construction of -200+125 deletion construct CCTTCCTTATGTTTGGCGTCTTCATGGGTGCGTCCCTCGGCTCCTGTA CAGCACTGG	59
AR: forward PCR primer for construction of -200+71 deletion construct: CCACAGGCAGAGGAGGCGACAGAGGCCATGGAAGACGCCAAAAACATAAAG AAAGGCC	60
AR: reverse PCR primer for construction of -200+71 deletion construct: CCTTCCTTATGTTTGGCGTCTTCATGGCCTCTGTCGCTCCTCTGCCCTG TGG	61
AR: forward PCR primer for construction of -200+50 deletion construct: GGGAGAGCAGGGACGGTCCGGAGCAAGCCCACCATGGAAGACGCCAAAAACA TAAAGAAAGGCC	62
AR: reverse PCR primer for construction of -200+50 deletion construct: GGCCTTCTTATGTTTGGCGTCTTCATGGTGGGTTGCTCCGGACCGTC CGCTCTCCC	63
AR wild type HLH-ds sequence: TGTTGCATTTGCTCTCC	64
AR wild type HLH-3 sequence: GCTCTCCACCTCCCAG	65
AR wild type 5' HP sequence: GGTGAGGGAGGGAGAAAAGGAAA	66
Her2 promoter sequence (Fig. 9)	67
PCR primer for amplification of a 2000-bp fragment of the human Her2 promoter 5'- GCA CGC GTA AGC TTC AGG CCC CAC AAA ACC TA-3'	68
PCR primer for amplification of a 2000-bp fragment of the human Her2 promoter 5'- CGC TCG AGC CAT GGC TCC GGC TGG ACC CGG CTG GG-3'	69
Her2 wild type repressor sequence downstream of the putative TATA box: GAATGAAGTT	70
Her2 wild type complex regulatory region: CGCTTGCTCCCAATC	71
Her2 wild type TATA box/ets site: GAGGAAGGTATAA	72
natural beta-lactamase promoter P3 GACGTCAGGTGGCACTTTGGGGAAATGTGCGCGGAACCCCTATTGTTTAT TTTCTAATACATTCAAATATGATTCGCTCATGAGACAATAACCCTGATAAATG CTTCAATAATA TTGAAAAGGAAGAGT	73
modified beta-lactamase promoter (BlaMT) promoter: GACGTAGGTGGCACTTTGGGGAAATGTGCGCGGAACCCCTATcTGTTTgTT CTTcTAgAcACATTCAcAcATGTATCCGCTCATGAGACAATAACCCTGATAAATGC TTCAATqAcATTGAqAAAGGAAGAGT	74
beta-lactamase promoter wild type sequence for the -35 region (-41 to -30, M6): AATACATTCAA	75
beta-lactamase promoter wild type sequence for the -10 region (-17 to -6, M8): CATGAGACAATA	76
beta-lactamase promoter wild type sequence for the start site (-5 to +7, M9): ACCCTGATAAAT	77
beta-lactamase promoter wild type sequence for the +20 to +31 site (M11): TTGAAAAGGAA	78

IT IS CLAIMED:

1. An isolated nucleic acid regulatory sequence for a cyclin D1 promoter, said regulatory sequence characterized by the ability to regulate expression of a gene  
5 operably linked to a cyclin D1 promoter containing said regulatory sequence.
2. The regulatory promoter sequence of claim 1, wherein said sequence is selected from the group consisting of SEQ ID NO.:5, SEQ ID NO.:6 and SEQ ID NO.:8.
- 10 3. An isolated nucleic acid regulatory sequence for a CD40L promoter, said regulatory sequence characterized by the ability to regulate expression of a gene operably linked to a CD40L promoter containing said regulatory sequence.
- 15 4. The regulatory promoter sequence of claim 3, wherein said sequence is selected from the group consisting of SEQ ID NO.:12, SEQ ID NO.:13, SEQ ID NO.:14 and SEQ ID NO.:15.
- 20 5. An isolated nucleic acid regulatory sequence for an HBV promoter, said regulatory sequence characterized by the ability to regulate expression of a gene operably linked to an HBV promoter containing said regulatory sequence.
6. The regulatory promoter sequence of claim 5 wherein said HBV promoter is a core, preS1 or X promoter.
- 25 7. The regulatory promoter sequence of claim 6 wherein said sequence is the HBV core promoter sequence presented as SEQ ID NO.:20 or SEQ ID NO.:21.
8. The regulatory promoter sequence of claim 6 wherein said sequence is the HBV preS1 promoter sequence presented as SEQ ID NO.:23 or SEQ ID NO.:24.
- 30 9. The regulatory promoter sequence of claim 6 wherein said sequence is an HBV X promoter sequence selected from the group consisting of SEQ ID NO.:26, SEQ ID NO.:27 and SEQ ID NO.:28.

10. An isolated nucleic acid regulatory sequence for a vancomycin-resistant enterococci (VRE) promoter, said regulatory sequence characterized by the ability to regulate expression of a gene operably linked to a VRE promoter containing said regulatory sequence.

5

11. The regulatory promoter sequence of claim 10 wherein said sequence is selected from the group consisting of SEQ ID NO.:32, SEQ ID NO.:33 and SEQ ID NO.:34.

10

12. An isolated nucleic acid regulatory sequence for an androgen receptor (AR) promoter, said regulatory sequence characterized by the ability to regulate expression of a gene operably linked to an AR promoter containing said regulatory sequence.

15

13. The regulatory promoter sequence of claim 12, wherein said sequence is selected from the group consisting of SEQ ID NO.:64, SEQ ID NO.:65 and SEQ ID NO.:66.

20

14. An isolated nucleic acid regulatory sequence for a HER2 promoter, said regulatory sequence characterized by the ability to regulate expression of a gene operably linked to a HER2 promoter containing said regulatory sequence.

15. The regulatory promoter sequence of claim 14, wherein said sequence is selected from the group consisting of SEQ ID NO.:70, SEQ ID NO.:71 and SEQ ID NO.:72.

25

16. An isolated nucleic acid regulatory sequence for a beta lactamase (Bla) promoter, said regulatory sequence characterized by the ability to regulate expression of a gene operably linked to a Bla promoter containing said regulatory sequence.

30

17. The regulatory promoter sequence of claim 16 wherein said sequence is the Bla promoter sequence presented as SEQ ID NO.77 or SEQ ID NO.78.

18. A vector comprising a promoter regulatory nucleic acid sequence of any one of claims 2, 4, 7, 8, 9, 11, 13, 15 and 17.

35

19. The vector of claim 18, wherein said vector is an expression vector comprising  
(i) said promoter regulatory nucleic acid sequence operably linked to a promoter and  
control sequences recognized by a host cell transformed with the vector; and (ii) a  
transgene encoding an autologous or heterologous gene product.

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20. The vector of claim 19, wherein said transgene is a reporter gene.

21. A host cell comprising the vector of claim 20.

10

22. The host cell of claim 21, wherein said host cell is a prokaryotic cell.

23. The host cell of claim 21, wherein said host cell is a eukaryotic cell.

24. The host cell of claim 21, wherein said host cell is a mammalian cell.

15

25. A method of regulating gene expression in a cell comprising

(i) introducing into a cell an expression vector according to claim 19,

(ii) exposing said promoter regulatory sequence to a cellular factor or a DNA  
binding compound resulting in modulated expression of said transgene; and

20

(iii) detecting the expression thereof.

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1/14

GCACGTGGCATGGAGACCACCGTGAACGCCAACAAAT  
 \*  
TGCCCAAGGTCTTACATAAGAGGACTCTGGACTCTCAGC  
 \_\_\_\_\_  
 HNF4  
 \*  
AATGTCAACGACCGACCTTGAGGCATACTTCAAA GACTGT  
 \_\_\_\_\_  
 HNF3-1  
 \*  
TTGTTAAAGACTGGAGGAGTTGGGGAGGAGATTAGGT  
 \_\_\_\_\_  
 3-2  
 \*  
 TAAAGGTCTTGTACTTAGGAGGCTGTAGG CATAAATTGGT  
 \* \* \* \*  
 CTGCGCACCCAGCATTGCAACTTTCACCTCTGCCTAA  
 \*  
 TCATCTCTTG  
 \* nucleotide conserved at >95% among 75 HBV strains

Fig. 1A

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2 / 14

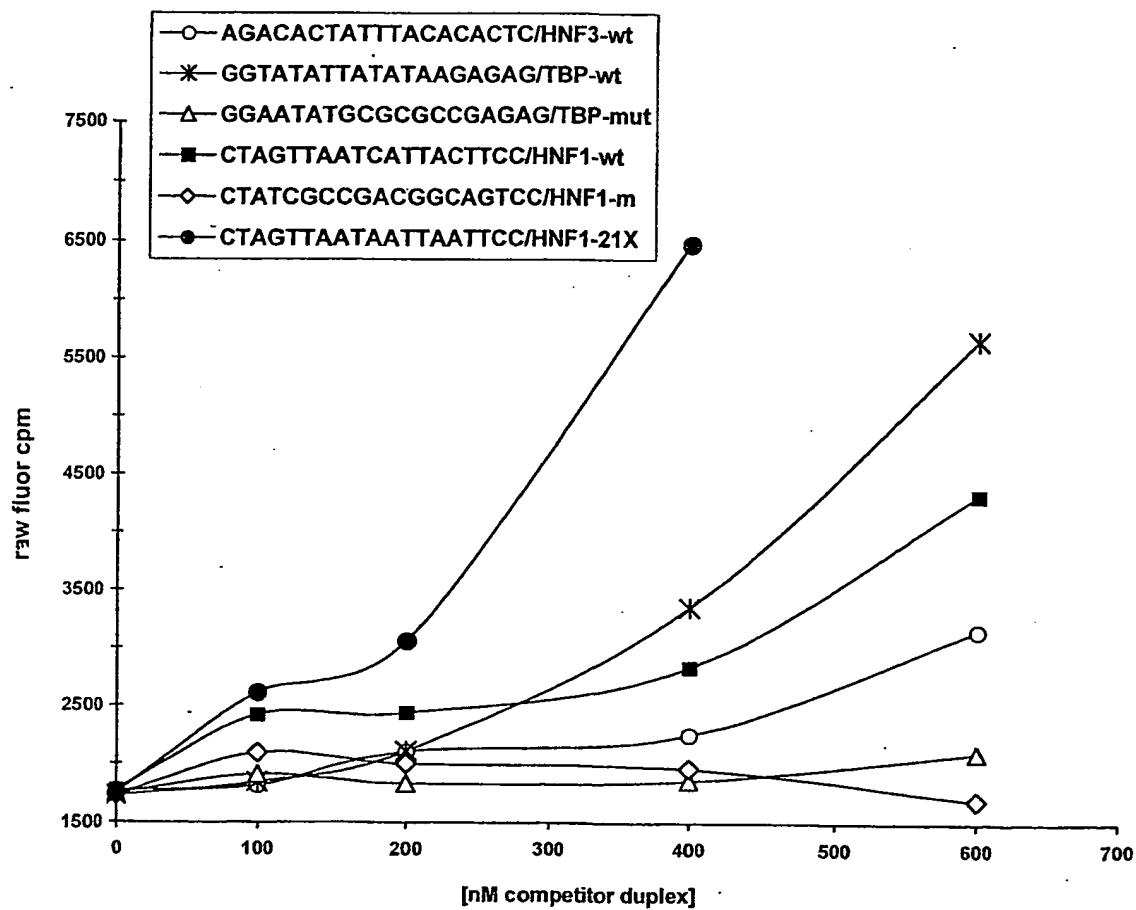
2761 ATGGAAGGCCGGTATATTATAAGAGAGAAACAACCATAGCGCCTCATTTGTGGGTC  
Sp1 TBP RNA Start

2821 ACCATATTCTGGGAACAAGATCTACAGC**ATGGGG**  
PreS1 protein start

Fig. 1B

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3/14

**Fig. 2**

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4/14

1081 CTA AGC AGG CTT TCA CTT TCT CGC CAA CTT ACA AGG CCT TTC TGT GTA AAC AAT  
NF1(1100-1119)

2c (1119-1134)

1135 ACC TGA ACC TTT ACC CCG TTG CCC GGC AAC GGC CAG GTC TGT GCC AAG TGT TTG  
EF-C(1148-1168)

1189 CTG ACG CAA CCC CCA CTG GCT GGG GCT TGG TCA TGG GCC ATC AGC GCA TCC GTG  
E(1180-1202) NF1(1209-1236) X-PBP(1229-1245)

1243 GAA CCT TTT CGG CTC CTC TGC CGA TCC ATA CTG CGG AAC TCC TAG CCG CTT GTT

1297 TTG CTC GCA GCA GGT CTG GAG CAA ACA TTA TCG GGA CTG ATA ACT CTG TTG TCC

1351 TAT CCC GCA AAT ATA CAT CGT TTC CAT GGC TGC TAG 1386

Fig. 3

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5/14

CAGCTGGG CCGCCCTTGT GCGCGGGCTG ATGCTCTGAG GCTTGGCTAT  
GCAGGGGGCCA ACGCGATTGT GGGTGCTCGG GGAGTGGGGG GGGGCACGAC CGTAGGTGCT  
CCCTGCTGGG GCAACCCATC GCTCCCCATG CGGAATCCGG GGTAATTAC CCCCCCAGGA  
CCCAGAATAT TAGTAATCCT AATTCCCCGC GGGGGAGGGG GCGCGGGAGG AATTACCCCT  
GAAAGGTGGG GGTGGGGGGG GTCGCATCTT GCTGTGAGCA CCCTGGCGAA GGGGAGAGGG  
CTTTTCTAT CAGTTTCTT TGAGCTTTA CTGTTAAGAG GGTACGGTGG TTTGATGACA  
CTGAACATA TTCAAAAGGA AGTAAATGAA CAGTTTCTT AATTGGGGC AGGTACTGTA  
AAAATAAAA CAAAAGTTAA GACAGTAAAA TGCCCTTTA TTTTTAATG CACCAAAGAG  
ACAGAACCTG TAATTTAAA AACTGTGTAT TTTAATTAC ATCTGCTTAA GTTTCGATA  
ATATTGGGA CCTCTCATG TAACCACGAA CACCTATCGA TTTTGTAA AATCAGATCA  
GTACACTCGT TTGTTAATT GATAATTGTT CTGAATTATG CCGGCTCC-TG CCAGCCCCCT  
CACGCTCACG AATTCACTCC CAGGGCAAAT TCTAAAGGTG AAGGGACGTC TACACCCCCA  
ACAAAACCAA TTAGGAACCTT CGGTGGCTTT GTCCCAAGGCA GAGGGGACTA ATATTTCCAG  
CAATTAAATT CCTTTTTAA TTAAAAAAA TGAGTCAGAA TGAGATCAC TGTTTCTCAG  
CTTCCATTG AGAGGTGTGT TTCTCCGGT TAAATTGCCG GCACGGGAAG GGAGGGGGTG  
CAGTTGGGA CCCCGCAAG GACCGACTGG TCAAGGTAGG AAGGCAGGCC GAAGAGTC  
CAGGCTAGAA GGACAAGATG AAGGAAATGC TGGCCACCAT CTTGGGCTGC TGCTGGAATT  
TTCGGGCATT TATTTTATT TATTTTTGA GCGAGCGCAT GCTAAGCTGA AATCCCTTA  
ACTTTAGGG TTACCCCCCTT GGGCATTGCA AACGACGCC CTGTGCGCCG GAATGAAACT  
TGCACAGGGG TTGTGTGCCG GGTCTCCCC GTCTTCGAT GCTAAATTAG TTCTGCAAT  
TTACACGTGT TAATGAAAAT GAAAGAAGAT GCAGTCGCTG AGATTCTTG GCCGCTGTC  
CGCCCGTGGG TGCCCTCGTG GCGTTCTGG AAATGCGCCC ATTCTGCCGG CTTGGATATG  
GGGTGTCGCC GCGCCCCAGT CACCCCTCT CGTGGTCTCC CCAGGCTGCG TGCTGCG  
GCCTTCCCTAG TTGTCCCCCTA CTGCAGAGCC ACCTCCACCT CACCCCTAA ATCCGGGGG  
ACCCACTCGA GGCGGACGGG GCCCCCTGCA CCCCTCTTCC CTGGCGGGGA GAAAGGCTGC  
AGCGGGCGA TTGCAATTTC TATGAAAACC GGACTACAGG GGCAACTCCG CCGCAGGGCA  
GGCGCGCGC CTCAGGGATG GCTTTGGGC TCTGCCCTC GCTGCTCCCG GCGTTGGCG  
CCCGCGCCCC CTCCCCCTGC GCCCCCCCCC GCCCCCTCC CGCTCCCTATT CTCTGCCGG  
CTTTGATCTT TGCTTAACAA CAGTAACGTC ACACGGACTA CAGGGGAGTT TTGTTGAAGT  
TGCAAAGTCC TGGAGCCTCC AGAGGGCTGT CGGCGCAGTA GCAGCGAGCA GCAGAGTC  
CACGCTCCGG CGAGGGGCAG AAGAGCGCA GGGAGCGCGG GGCAGCAGAA GCGAGAGCCG  
AGCGCGGACC CAGCCAGGAC CCACAGCCCT CCCCCAGCTGC CCAGGAAGAG CCCCCA

Fig. 4

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6/14

10	20	30	40	50	60	70
GAATTCACTG GGGAGAGCAT TCAGGAAGAT GACAACAGGA TAATAGGTCA ACAGAGTAAT AGAGAGGTGC						
CTTAAAGTGCAC CCCTCTCGTA AGTCCTTCTA CTGTTGTCCT ATTATCCAGT TGTCTCATT A TCTCTCCAGC						
80	90	100	110	120	130	140
CTAAAAATAA ACTCTAAGAA GTATTCAAGCC AAAACTATTA TTGAGCTAAT ATGGTGGGA TCAATTTCAG						
GATTTTATT TGAGATTCTT CATAAGTCGG TTTTGATAAT AACTCGATTA TTACACCCT AGTTAAAGTC						
150	160	170	180	190	200	210
GGGAATATTG TGGGCAGAAG TCAGACTGTA GGAGGCTGGG GATCAAGAAG TTGAGGCAAG GAGGTTGGAC						
CCCTTATAAC ACCCGTCTTC AGTCTGACAT CCTCCGACCC CTAGTTCTTC AACTCCGTTC CTCCAACCTG						
220	230	240	250	260	270	280
AACAACTGTT TTTCAAGTT GGTCACTGTA ACAAACTCTG GACCTTCAGC CTCCCCCTCCC TCAGGGTCTTG						
TTGTTGACAA AAAAGTTCAA CCAGTGCACT TGTAGACAGA CTGGAAAGTCG GAGGGGAGGG AGCCCAGAAC						
290	300	310	320	330	340	350
GCTGAGCTGA TTGCAGGGCC CCTGCAGCTC TGGCACTCTC AAGTTGTATA AAACGTGACAG TGCAGAAGTC						
CGACTCGACT AACGTCCCGG GGACGTCGAG ACCGTGAGAG TTCAACATAT TTTGACTGTC ACGTCTTCAG						
360	370	380	390	400	410	420
CTTGAGCCCA TTTGGCTCT CATGATAATT TTCCCTTCAGT GGAACATAAGG TTACTTGCT AAGAACCAAA						
GAAACTCGGGT AAAACCGAGA GTACTATTAA AAGGAAGTCA CCTTGATTCC AATGAACAGA TTCTTGGTTT						
430	440	450	460	470	480	490
GCCTCTGACT TGACTGATCA AAGTTCATCA CGTGCATCGA AGCCACCTAC TTGGCAGATG TAGTGAAAAG						
CGGAGACTGA ACTGACTAGT TTCAAGTAGT GCACGTAGCT CGGGTGGATG AACCGTCTAC ATCACTTTTC						
500	510	520	530	540	550	560
CTACATAGAT CTGGGCCAG GACAGGATGC TGGGGCGTGG GAGGGGAAGA AAGCAGGTGC TAACTATATA						
GATGTATCTA GACCCGGTC CTGTCCTACG ACCCCGCACC CTCCCCCTCT TTCTGCCACG ATTGATATAT						
570	580	590	600	610	620	630
GATAGCATGC CTATCAGAGC AGTTTTTACG TTTCCTATTT GTCTCTCAAA ACAATTTTAT AGGAATCATC						
CTATCGTACG GATAGTCTCG TCAAAAATGC AAAGGATAAA CAGAGAGTT TGTAAAATA TCCTTAGTAG						
640	650	660	670	680	690	700
AAAGCAATT TATCATGGTT TCTAGACCAG GTTGGATGT GAGGTAGGG A TTCCACAGC TGCTTTTAGT						
TTTCGTTAAA ATAGTACCAA AGATCTGGTC CAAACCTACA CTCCATCCCT AAAGGTGTCG ACGAAAATCA						
710	720	730	740	750	760	770
TTGAAGGAAA TCTGATAAGA TGATGCAAAA GCCCTTCAGA AATGTGTAAT CCTACACACT TCAGTGATTC						
AACTCCCTT AGACTATTCT ACTACGTTT CGGGAAAGTCT TTACACATTA GGATGTGTGA AGTCACTAAG						
780	790	800	810	820	830	840
AATTCAATTG CAAAACCTAA GGTGTTTTA ATATTGTTAT TGTTCATTTG GTTTTACCA ACATGTAAGG						
TTAAGTAACA GTTTGAATT CCACAAAAAT TATAACAATA ACAAGTAAAC CAAAATGGT TGTACATTCC						
850	860	870	880	890	900	910
AGTTGGCAAT TATTTGTTAA ACTCATGTCT TAGGCTAAAT AAATCCAAA AAATTCAGGA TGAGAATTGT						
TCAACCGTTA ATAAACAATT TGAGTACAGA ATCCGATT A TTAAGGTTT TTAAGTCCT ACTCTTAACA						

**Fig. 5A**

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7/14

920            930            940            950            960            970            980  
 TTATTGCTTA ACGTGTGTCA AATTCTTCC ATGCACATCT TTATTAGATC TTCACAGCAA CCTACAGGAT  
 AATAACGAAT TGCACACAGT TAAAGAAGG TACGTAGA AATAATCTAG AAGTGTGTT GGATGTCCTA  
  
 990            1000          1010          1020          1030          1040          1050  
 AAGCAAGACA GGTGCAAGTG CCTCCCTTGG GTATGAGGAA ACTGAGGTCT AAAGAGATGA AGTGATTGC  
 TTCGTTCTGT CCACGTTCAC GGAGGAAACC CATACTCCT TGACTCCAGA TTTCTCTACT TCACTAACG  
  
 1060          1070          1080          1090          1100          1110          1120  
 CCAAGGCTCA TAGCAATTAA TTGGTAGAGC AAAGACTAGA ATTCTCTTAA CTGCAGCCTA TTTCCCTAT  
 GGTTCCGAGT ATCGTTAAAT AACCATCTCG TTTCTGATCT TAAGAGAATT GACGTCGGAT AAAAGGGATA  
  
 1130          1140          1150          1160          1170          1180          1190  
 TCTGAAGTGT TACATCAGCA TCAACAATTAA TCTAATGGAT TGGAACAGTG TACACAGGCA GCTTAGCTAC  
 AGACTTGACA ATGTAGTCGT AGTTGTTAAT AGATTACCTA ACCTTGTACAC ATGTGTCCGT CGAATCGATG  
  
 1200          1210          1220          1230          1240          1250          1260  
 GTCAAGTCAC GATTTTACT TIAACTTCAA TTCCAGAGTC TTGGCCTGAT TTCCCTCAAG ACCCTACTTA  
 CAGTTCAGTG CTAAAAATGA AATTGAAGTT AAGGTCTCAG AACCGGACTA AAGGGAGTTC TGGGATGAAT  
  
 1270          1280          1290          1300          1310          1320          1330  
 TCTTTGGCTT TGGAAAATTAA TTTTTCTTG CATTATCTTT CCAGCTAAAT TTTATTTAAT AACCATCAGC  
 AGAAAACCGAA ACCTTTAAAC TAAAAGAAC GTAATAGAAA GGTGATTAA AAATAAATTA TTGGTAGTCG  
  
 1340          1350          1360          1370          1380          1390          1400  
 ATGCTTTTTTG TGCTTATGCA CATGTAGACT TGACCTGAAA ACCTGCCAGG CTTTCATTGA GTTTAGTGAT  
 TAGAAAAAAA ACGAAATACG GTACATCTGA ACTGGACTTT TGGACGGTCC GAAAGTAACT CAAATCACTA  
  
 1410          1420          1430          1440          1450          1460          1470  
 TAAAGAAGTA AAGTTCTGAG AAGCAATTAG TTGATGGGAC ACCAGTCATA AAATCAATCC AAACCTTTGT  
 ATTTCTTCAT TTCAAGACTC TTCTGTTAAC AACTACCCCTG TGGTCAGTAT TTTAGTTAGG TTTGAAAACA  
  
 1480          1490          1500          1510          1520          1530          1540  
 TGACATGTGT TTCTTCTCC ATATACCAGG TTCCCGCTTC GTATTAGTAA GATTGAAATT GAAATAAGTC  
 ACTGTACACA AAGAAAGAGG TATATGGTCC AAGGGCGAAG CATAATCATT CTAACCTTAA CTTTATTCA  
  
 1550          1560          1570          1580          1590          1600          1610  
 TATTGCTGGT GGATGAATTG GTCACTTCC TTGAAACTGG TGAACCCAAA AAGTTAGACA GTGATAGGAA  
 ATAACGACCA CCTACTTAAAC CAGTGAAGG AACTTTGACC ACTTGGTTT TTCAATCTGT CACTATCCTT  
  
 1620          1630          1640          1650          1660          1670          1680  
 AATACTGCCA TTGTCTGTTA AGAAGTCTAT GACATTTCAA GGCAAGAATG AATATATGGA AGAAGAACT  
 TTATGACGGT AACAGACAAT CTTCAGATA CTGTAAAGTT CCGTTCTTAC TTATATACCT TCTTCTTGA  
  
 1690          1700          1710          1720          1730          1740          1750  
 TGTTTCTCT TTACTTACAA AAAGGAAAGC CTGGAAAGTGA ATGATATGGG TATAATTAAA AAAAAAAA  
 ACAAAAGAAGA AATGAATGTT TTCTCTTCA GACCTTCACT TACTATACCC ATATTAATT TTTTTTTTT  
  
 1760          1770          1780          1790          1800          1810          1820  
 AAAACAAAAA ACCTTACGT AACGTTTGC TGGGAGAGAA GACTACGAAG CACATTTCC AGGAAGTGTG  
 TTTGTTTTT TGGAAATGCA TTGCAAAACG ACCCTCTTCTT CTGATGCTTC GTGAAAAGG TCCTTCACAC

Fig. 5B

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8/14

1830        1840        1850        1860        1870        1880        1890  
 GGCTGCAACG ATTGTGCGCT CTTAACTAAT CCTGAGTAAG GTGGCCACTT TGACAGTCTT CTCATGCTGC  
 CCGACGTTGC TAACACGCGA GAATTGATTA GGACTCATTC CACCGGTGAA ACTGTCAGAA GAGTACGACG  
  
 1900        1910        1920        1930        1940        1950        1960  
 CTCTGCCACC TTCTCTGCCA GAAGATACCA TTTCAACTTT AACACAGCAT GATCGAAACA TACAACCAAA  
 GAGACGGTGG AAGAGACGGT CTCTATGGT AAAGTTGAAA TTGTGTCGTA CTAGCTTGT ATGTTGGTT  
  
 1970        1980        1990        2000        2010        2020        2030  
 CTTCTCCCCG ATCTGCGGCC ACTGGACTGC CCATCAGCAT GAAAATTTT ATGTATTTAC TTACTGTTTT  
 GAAGAGGGGC TAGACGCCGG TGACCTGACG GGTAGTCGTA CTTTTAAAAA TACATAAAATG AATGACAAAA  
  
 2040        2050        2060        2070        2080        2090        2100  
 TCTTATCACCA CAGATGATTG GGTCAAGCACT TTTTGCTGTG TATCTTCATA GAAGGCTGGGA CAAGGTAAGA  
 AGAATAGTGG GTCTACTAAC CCAGTCGTGA AAAACGACAC ATAGAAGTAT CTTCCGACCT GTTCCATTCT  
  
 2110        2120        2130        2140        2150        2160        2170  
 TGAACCACAA GCCTTTATTA ACTAAATTG GGGTCCTTAC TAATTCTAG GTTGGTTCTA CCCAAATGAT  
 ACTTGGTGTG CGGAAATAAT TGATTTAAC CCCAGGAATG ATTAAGTATC CAACCAAGAT GGGTTTACTA  
  
 2180        2190        2200        2210        2220        2230        2240  
 GGATGATGGT AGAAACCAA TAGAAGAATG GTCTTGTGGC ATAATGTTTG TTCCCTAGTC AATGAACTCT  
 CCTACTACCA TCTTGGTTT ATCTTCTTAC CAGAACACCG TATTACAAAC AAGGGATCAG TTACTTGAGA  
  
 2250        2260        2270        2280        2290        2300        2310  
 CATATTCTTG TCTCTGGTTA GGATCTTGGG ATCTGGACTC AGACTGCCTG GGCTCAAATC TTGGCTCTGC  
 GTATAAGAAC AGAGACCAAT CCTAGAACCC TAGACCTCAG TCTGACGGAC CCGAGTTAG AACCGAGACG  
  
 2320        2330        2340        2350        2360        2370        2380  
 CCATACCATC TCTGTTATCC TGGGGCAAGT GCCTCAGTT CCACATCTGA GAAATGGGGA TGGTAGTGGT  
 GGTATGGTAG AGACAATAGG ACCCCGTTCA CGGAGTCAAA GGTGTAGACT CTTTACCCCT ACCATCACCA  
  
 2390  
 GTCCATTCA TAGAT  
 CAGGTAAAGT ATCTA

Fig. 5C

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9/14

GAGATGTATATAATTTTAGGAAATCTCAAGGTTATCTTACTTTCTTA  
GGAAATTAACAATTAAATTAAGAACGGCTCGTTCTTACACGGTAGACTTA  
ATACCGTAAGAACGAGCCGTTTCGTTCTTCAGAGAAAGATTGACAAGATTA  
CCATTGGCATCCCCGTTTATTTGGTGCCTTCACAGAAAGGGTTGGTCTTAA  
TT

**Fig. 6**

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10 / 14

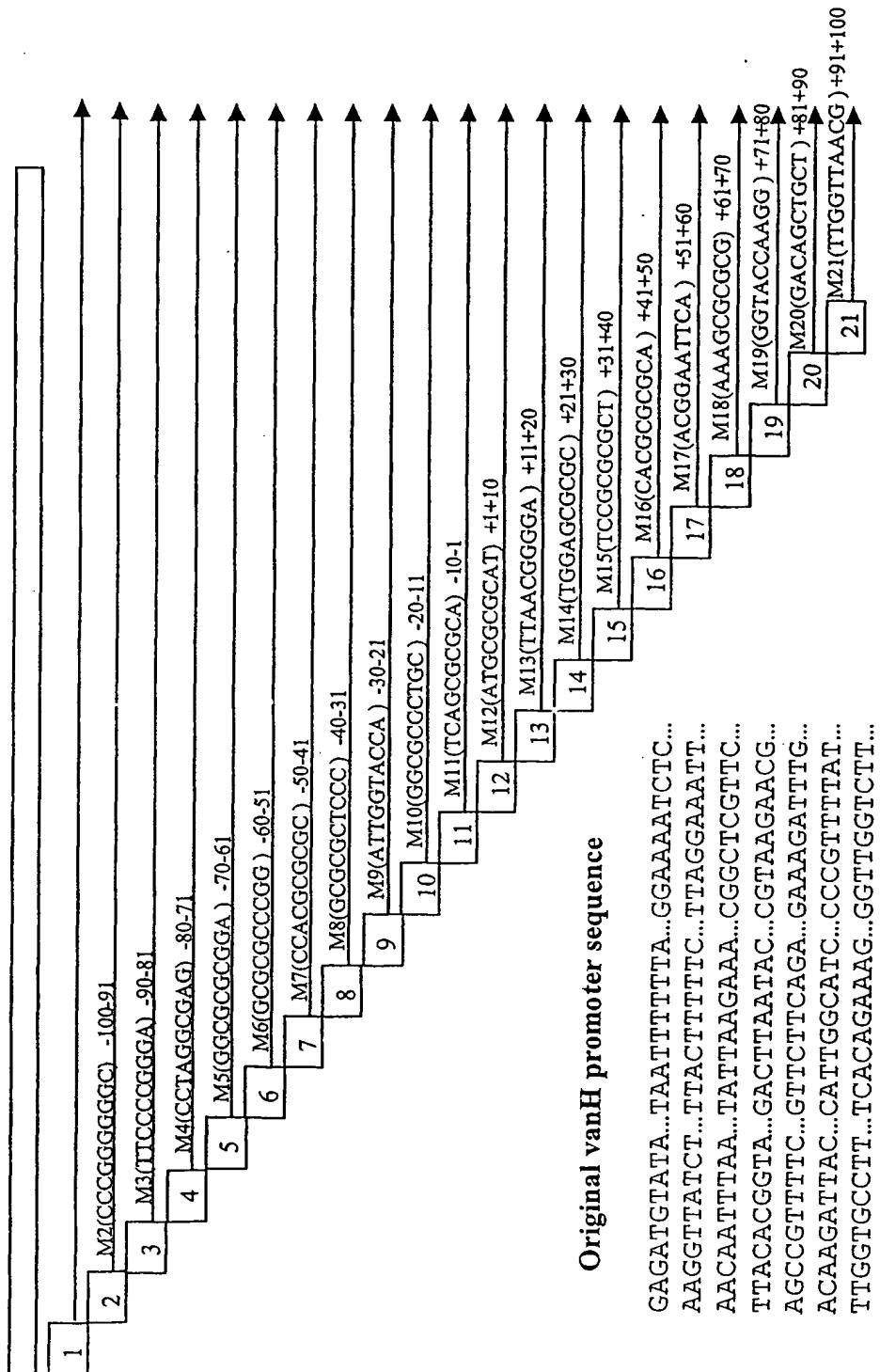


Fig. 7

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11/14

TCTAGAAAAT AATTCCCAAT ATTGAATCCC AAAGAATTCA ACATTGGGC TGTGTTTGA 61  
 AAGATAAGTT GAATTGGTC ATGAAGGAG AGAGGGGGGA TACAATTCA GTAAAAGGTA 121  
 ACAGCAAGGT CCAAAGACAG TCAGGTCTC AGTAGTATGG AGTATATTCA GAGGGAGCCA 181  
 AGATGCTGA TGTGAACAA AAAGATTGGT GGTTGGTAGG AGGAAGAGGT GTGAGAAGAG 241  
 GCTGAAAGA AAAATTGAAA CTTGATTGTG ATGGACTTA AAGGCTAGGC TATGGGACTT 301  
 GGACATGAAT CTGCAGGCCA GTGTTGCAG ACTGGCGCC ATAACHTGCT ATCACAGCAA 361  
 CACAGACATG TGTTGTTGG CCTGCAGAGG TTTGGCCTGC ATGATGATT TAAACCATCT 421  
 GAATTAGTAG CCATCATTAA CAAAATCAA GAGATGCCAC ATTAAAATAT GGAATGCTGC 481  
 TGTTCTGAA AATAATGAAA CATCTGAAAC ATTGAGGCCA CATTCCGTAC TGACAGCAAT 541  
 CAGTTGGAGC TGGTAGTGA CTGCCACTT TACATGGGC ATCTGATCCC TAGTCGATTA 601  
 CAGCTGCCAC CACTTCCCT TATCTCTCA ATACCAAGCT CTTTCACTC ATTGTTGTTA 661  
 CTTAAGAGAT ATTTGGTTT GAAACCTG ATGCAGGTA TTGAGGGTTA TAGAGCAGAG 721  
 GACAGATGCT ATCAGAGTTG TCTTTAAGA AAGAACCTC TGTCTTCAT TTTGTTGAAG 781  
 ATAGCCTGGA AGAGGGCAGC CAGGGAGAA GTTGGGCTG GAGCTATGAG AAAGCATAAG 841  
 ATGAGATGAT GGCTTCAACA TTGAGGACAG AAAGAATATT GAGATGAGAA AGTAGTCCAT 901  
 ATAAGCATCT ATGCAAAGGA AATAGCAGAT GTCTCAAAT CAGCAGAGGC AACAACTCTG 961  
 AAAGTTTATT CATAAGCCCC TCTTTCATC TCCAATCCAG TTCAAATGTA ATTATTTAAA 1021  
 TTGTTCTCA CTCTCCCTC TGGATCATGA ATGAGCTCCT TAAATGCAGG GTCCACAGTG 1081  
 TCCTATTCTC CAGTGAATT CAAGTGCCTA GCACAGAGCC TGGCAAATAG TAAATGCTTA 1141  
 ACAAAATATTC GTTCAGTGC TGAATTGGAG TGATTCTCA CTTTGCCTCA TAAGTTGAAA 1201  
 AAAGGTTTAT TACATACCTA AATATGCTGA AATCACAGGG CATTGGCAA CCCCCCAAAA 1261  
 CCAAAACTCC CAGTTGGAA ACAGAATTAA TATTCTGTGA AAATAAAATC CATTCACTTA 1321  
 TTCAAAAAAT ATTTATTTAA CAATGACCAT GTCCACACCA GGCTGAGTCC TAAGGATTCA 1381  
 ATGATGAACA AAAACCAACA TGATTCTGC TCTTAGGAAA CATACTGTT AGTGAAGGAAA 1441  
 ACAGATTGTC AGAAGTCCCT CAACAAATAC TGGGTGCTAT TAAAATATAT TAAAAGGTGA 1501  
 GTGGGTGAGG GACTTGAGCT AGCTTGTG TTTCAGGAAG TCTTCCTGGA TGTGCTGATA 1561  
 TGCATAGGCA TTAACTAGAT AATAGAGAG AAGGATGAAAC CAACATGCA GGTAGAGGGA 1621  
 ACAGAATATG CAAAGGCAGG AAGGATTATG GAGTCGTTGG AGGAACCTGAA TAAAGGCCA 1681  
 GTGTAAGTGG ATCTCAGAAA ACAGGAGGAA AGGTGTATGA GATGAGATCA GAGAGGCAGA 1741  
 TCATGTGGGG TATGGTTAAT GTTTGGACT TTCTTATTAA GAGCAATGGG GAGACAGTGA 1801  
 CAGGACTTAA ACGGGGAAAT AATATGACCA GATTAAACTT TCTAAAAAAC CCTCTATGCA 1861  
 AATATATATT GAGGTTAAT TATTGACAA GATTCAAAGG CAACAAAGTG GAGAGAGAAT 1921  
 AGTATTTCTA AAAATGGTG CAAACAAAT AGGACATCTA TATTAAAAGT TGGGTATCTG 1981  
 TCTACAAAC TTAATTCAAA ATGGATCACA GACCTAAATG TAAAACGTAA AGCTATACAA 2041  
 CTTCTGGAA GAAAACACAG ATGGAATCT GTGTGATCTT GAGTTGAAA ATGATTTATT 2101  
 ATATCTGACA CCATAATCCG TAAGTTAAC TAATTCTAA GTGAAACAAAG TGATGAACTG 2161  
 GACTTCATCA GAATTAAAAA TGTTGTGCT TCAAAAGACA CTGGTATGAT AATGAAGACA 2221  
 AACTACAGAT AAGTATTTGT TGAATCATAT TTCTGTAAAG GGAATTGTGG CTCAGAATAC 2281  
 ATAACCTCAA ACCCCCCATAA TAAATTACAA GTAGCCAAAT TAAAAAAAAA AAAAGAGAAA 2341  
 AAATTTCAG TCTTCATCAA AGAAAGTATC AATTGAAAAA TAAGCACATG AAAATGCTC 2401  
 TGCATCTTTA TTGATGGGG GATGAAATAA AAATTAAATG GGAAAGACAC CTCTAATTAG 2461  
 AATACTAAAAA TTAAAAAAGAC TGACCATACC AAGTATTGGT GAAAGTGGAAA TGAAAATGA 2521  
 TACAATCAAC TTAGGTAGAT GATTGGAGA TTTCTTACAA AAGTAGGTGT ATACCTACCC 2581  
 TGTGACTCAC CCATTCCATG GCTAAGTATT TACCTGAGAG AAATGAAAGA ATACATCCAT 2641  
 ACAAAAGATGT TTATACAAAT ATTATAGCA GTTTTATTG TAGTAGCCCC AAACGTGAAAA 2701  
 GAACCCAAAT GTCCATCAA AGTGAATGGA TAAACAAAGC GTGGTACAGC AATGCAATAG 2761  
 AATACTACTT AGCAATAAAG AAGAATGAGC TAGTGTATATA CATAACAGCT TAAATGTACA 2821  
 TCAAAGGCAT TGTGCTCAGT GAAAGATGCA AGTAAAAAAA AAAAGAGTA CATGCTGTAT 2881  
 AGTTCCATTG ACATAAAACT CTGAAAGATG AAAAACAGTC TATACTGACA GAAAGCAGAT 2941  
 CATTGGTTGC CTGAGGAGGA GGAGTATAGG AGAGGTGGAG GGAAAATGTA CAAAGTGGCA 3001  
 CAATAAAAAC TTTGGAATC ATAGATATAT TCACTATCTT GATTGAGTGA TGATTTCATG 3061

Fig. 8A

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12/14

AGTGCACGTG CGTGTGTCAA AAATGATCAA TTTATGCAAC TTTAAATATG TCCAGTTAT 3121  
 TGTATATATC AATTATACCT CAGTACGGCT ATTAAAAAGA AACCCCTCTGG CTGCACAATG 3181  
 CAGAACTGAT TCTAGGAAAG AGTGGAGGGA GGATGACCCT TTACAGTGCT CCAGGTGGAA 3241  
 GAGAACGGTG CCTTCTGGAA GTGAACCTAGG TTGGCAACAA CAGAGATGAA ATAAATGGGC 3301  
 AGATGTGTGA GATACTTAGG AAATAAAACCG CGATGGTCAC CATTTCACCA AGGTCAGCTC 3361  
 ATCCTGGCTT TCCAGAGCAA AGAGCTAGGG AAGACTTTAT TAATAATCC CTCTTGAAGT 3421  
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Fig. 8B

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Fig. 8C

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**Fig. 9**

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&lt;120&gt; PROMOTERS FOR REGULATED GENE EXPRESSION

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